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THE MECHANISM OF VITAMIN D MEIDATED PROTECTION FROM EXPERIMENTAL COLITIS

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THE MECHANISM OF VITAMIN D MEIDATED PROTECTION FROM EXPERIMENTAL COLITIS

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To my family, for their love and support.

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LIST OF ABBREVIATIONS

1,25(OH)2D	1,25 dihydroxyvitamin D
CYP27B1	1α-hydroxylase
TNBS	2,4,6-trinitrobenzenesulfonic acid
CYP24A1	24-hydroxylase
25(OH)D	25-hydroxyvitamin D
AIEC	Adherent-Invasive E. coli.
ADCC	Antibody-Dependent Cell-mediated Cytotoxicity
APC	Antigen Presenting Cell
AMP	Anti-microbial Peptide
AOM	Azoxymethane
cAMP	Cathelcidin
CDAI	Crohn’s Disease Activity Index
CD	Crohn's Disease
DC	Dendritic Cell
DSS	Dextran Sulfate Sodium
DNBS	Dinitrobenzene Sulfonic Acid
GI	Gastrointestinal
GWAS	Genome-Wide Association Studies

IgA	Immunoglobin A
IgG	Immunoglobin G
IBD	Inflammatory Bowel Disease
KO	Knockout
Mϕ	Macrophage
MHC	Major Histocompatibility Complex
mDC	Myeloid Dendritic Cell
Th0	Naïve T Helper Cell
NOD2	Nucleotide-binding Aligomerization domain-containing protein 2
TPA	Phorbol ester 12-O-tetradecanoylphorbol-13-acetate
pDC	Plasmacytoid Dendritic Cell
PUFA	Polyunsaturated Fatty Acids
RXR	Retinoid X Receptor
TJ	Tight Junctions
TLR	Toll Like Receptor
TER	Transepithelial Electrical Resistance
TFF3	Trefoil Factor 3
UC	Ulcerative Colitis
IOM	US institute of Medicine
VDR	Vitamin D Receptor
VDRE	Vitamin D Response Element
DEFB4	β-defensin 2

ABSTRACT

Wang, Fa. Ph.D., Purdue University, August 2016. The Mechanism of Vitamin D Mediated Protection from Experimental Colitis. Major Professor: Dr. James Fleet.

Inflammatory bowel disease (IBD) describes chronic and relapsing digestive system inflammation and affects over 1.5 million people in the US. Current treatment strategies for IBD are expensive and often lead to unsatisfactory outcomes. Therefore it is essential to understand the mechanism of IBD progression and develop prevention strategies. Epidemiological evidence showed that IBD risk is negatively associated with vitamin D status. This is consistent with the clinical observation that people with vitamin D deficiency have increased IBD severity. To establish the causal link between vitamin D status and IBD development, our lab used dextran sulfate sodium (DSS) to induce experimental colitis in mice which were fed either a high or low vitamin D diet. We found that low vitamin D intake caused more severe body weight loss and spleen enlargement in mice. Since colitis development is regulated by both colon epithelial cells and infiltrating immune cells, our next research goal was to separate the vitamin D effect on the two cell compartments and investigate their independent roles in the development of experimental colitis. To test the role of vitamin D signaling in colon epithelial cells, we developed a DSS inducible, large intestine specific transgenic mouse model, which enabled the examination of vitamin D receptor (VDR) function in colon epithelial cells during DSS-colitis. We found that

deleting VDR in colon epithelial cells (CAC; VDR KO) caused more severe colon damage but did not affect colon mucosa healing, immune cell activation or systemic colitis response (i.e., body weight loss, disease activity and spleen enlargement). To test the role of VDR in non-epithelial cells (primarily immune cells) during DSS-colitis, we used HV2; VDR KO mouse model non-epithelial cell VDR deletion. We demonstrated that deleting VDR from non-epithelial cells caused more robust colitis in both local and systemic levels, indicating that VDR in non-epithelial cells plays a more important role in colitis prevention than VDR in colon epithelial cells. We further tested the colonic cytokine profile in both CAC; VDR KO and HV2; VDR KO mice and found delayed healing corresponding to elevated M1 macrophage (Mϕ) activity. Therefore, we hypothesized that vitamin D can directly regulate Mϕ phenotype during colitis. This hypothesis was supported by a series of in vitro experiments using murine peritoneal Mϕs, which showed that 1,25(OH)2D treatment inhibited M1 while facilitating M2 Mϕ polarization and phenotype switching. The major finding of my thesis work is that vitamin D signaling protects against experimental colitis in mice through the primary effect of regulating Mϕ phenotype and the secondary effect of colon epithelial cell VDR response. Further experiments are needed to investigate the upstream regulation of vitamin D on monocyte- Mϕ lineage response during colitis.

CHAPTER 1. LITERATURE REVIEW: THE ROLE OF VITAMIN D IN INFLAMMATORY BOWEL DISEASE

1.1 Introduction

Inflammatory bowel disease (IBD) is a worldwide health issue and is commonly diagnosed in western countries, such as North America, North Europe and Australia (*1, 2*). The term IBD describes chronic and relapsing inflammatory response in the intestine, and can be further categorized as Crohn's Disease (CD) and Ulcerative Colitis (UC) (*3*). A recent report by the Center for Disease Control (CDC) estimated that there are 1-1.3 million people in US with IBD. The number of new diagnosed cases each year ranges from 3.1 to 14.6 cases per 100,000 people for CD and from 2.2 to 14.3 cases per 100,000 people for UC (CDC report). The average annual cost of IBD management is over $25000 per patient. If uncontrolled, IBD will also cause more severe health issues (e.g. colon cancer, cardiovascular diseases, etc.) and increased death rate (*4, 5*). For example, the mortality risk of CD patients is increased by up to 50% compared to healthy population (*6-8*). So far, medication can only alleviate the disease symptoms to a certain extent but not completely eliminate the disease development.

Life style factors influence human IBD risk and disease development. Since the late 20th century, IBD incidence in newly industrialized Asian countries has dramatically increased and is currently higher than that of the Western countries (*9*). This increased IBD incidence may due to Westernized lifestyle behaviors (smoking, stress, physical

activity etc.), environmental factors (e.g. pollution), and diet (e.g. high fat and low fiber) (*10*). One of the dietary factors, vitamin D, has been proposed to protect against IBD (*10*). This hypothesis has been supported by the evidence that IBD patients are often diagnosed with vitamin D deficiency, and serum 25-hydroxyvitamin D (25(OH)D) level is negatively correlated with CD severity (*11*, *12*). Multiple cell types in the inflamed intestine are potential vitamin D regulating targets, but the mechanisms have not been fully understood. This review will first introduce the current understanding of IBD development and management. It will then focus on the potential mechanisms of vitamin D mediated protection against IBD through regulating immune response and colon epithelial cell function.

1.2 Overview of IBD

1.2.1 Definition of IBD

IBD refers to chronic and relapsing inflammatory response in the gastrointestinal tract, and it can be further categorized as CD and UC (*3*). IBD patients suffer from gut inflammation that causes local and systemic symptoms, including colon ulceration, gastrointestinal bleeding, diarrhea, anemia, abdominal pain, and weight loss among others (*13*). CD and UC have different characteristics based on the disease location and histological phenotypes. In CD patients, histological damage can be observed as skip lesions at any location through the gastrointestinal (GI) tract. The damage often extends to the transmural layer which will further result in fibrosis and obstruction in the late stages of CD (*14*). UC, however, is mostly diagnosed in the large intestine, including the proximal colon, distal colon and rectum (*15*). The damage and inflammation is often

limited to mucosa without the involvement of deeper layers of the colon wall. IBD significantly reduces the quality of life for patients and increases the risk of developing other diseases, such as colon cancer and heart failure (*4, 5*). Current strategies for IBD management are expensive and the outcomes are not satisfied (*16*). Therefore, it is of urgent importance to understand the pathogenesis of IBD and develop novel prevention and treatment strategies against the disease.

1.2.2 IBD is a complex disease with multiple risk factors

IBD is a complex disease resulted from the interaction with multiple factors, including genetics, life-style, infection, and immune system disturbance (*13*). All of these factors, individually or combined, may affect disease initiation, development, recovery and relapse.

Genetics and IBD. Individuals with IBD family history have 8-10 times higher risk to develop this disease, which suggests the heritable potential of IBD (*17*). Epidemiology studies also showed that genetic background was associated with IBD risk. For example, individuals of European descent have higher risk of developing IBD (*18*), even though this may also be due to environmental influences and other confounding factors. One of the earliest genes identified to have a direct impact on CD susceptibility is Nucleotide-binding oligomerization domain-containing protein 2 (NOD2), which encodes a plasma pathogen recognition receptor involved in innate immune response regulation and anti-microbial function (*19*). Individuals with NOD2 gene mutation also have increased IBD risk. Starting from the early 2000's, genome-wide association studies (GWAS) have been conducted across populations, and until 2013, numerous candidate genes within 163 loci have been linked to IBD (*20*). The concordance rates of IBD in twins have been

analyzed to estimate the contribution of genetic factors to IBD. The concordance rates of monozygotic and dizygotic twins in CD (30.3% and 3.6%) are higher than those in UC (15.4% and 3.9%) (*21*). This indicates that genetic factors contribute more to CD development than to UC. In addition, it also suggests that factors other than genetics induce disease development (*22*).

Lifestyle and IBD. IBD incidence has increased with industrialization in developing countries, indicating life-style factors may influence disease development. As early as the 1950s, IBD was mostly recognized and diagnosed in North American and North Europe, and so far the IBD prevalence is still the highest in developed countries (*1, 2*). Interestingly, IBD incidence was significantly increased in newly industrialized countries starting from the late 20th century (*23*). Because of this, Westernized life-styles, including smoking, urban setting, obesity, stress and diet, have been associated to increased IBD risk (*24*). Among those, dietary factors have attracted the most attention. Observation studies showed that people having high fat diet were more likely to develop IBD, whereas those consuming a diet containing high fiber, fruit and vegetables had lower IBD risk (*25*). More specifically, n-6 polyunsaturated fatty acids (PUFA) rich meat, such as red meat and cooking oils, has been linked to increased UC risk, whereas n-3 PUFA is healthier (*26*). Vitamins and minerals are also associated with IBD pathogenesis. For example, dietary vitamin D has been proposed to have protective effects against IBD through its immune regulation function (*27*). IBD patients are often diagnosed with vitamin D deficiency, especially those with active and severe IBD symptoms (*28*). From the treatment aspect, vitamin D supplementation raised up vitamin D status in IBD patients and meanwhile improved IBD disease outcomes in clinical trials (*28*, *29*).

Infection, gut flora and IBD. The human gut contains as many as 10^{14} bacteria cells, and some of them are associated with gut homeostasis and IBD development (*30, 31*). For example, salmonella and campylobacter infections have been associated with higher risk of IBD (*32*). *Paratuberculosis* is also suggested to induce CD but the results among studies are not consistent (*33, 34*). In contrast, parasite infection (e.g. Helminth) protects against IBD, and the proposed mechanism is through parasite induced non-classical macrophage (Mϕ) polarization and activation (*35*). Not only pathogens but also unbalanced commensal bacteria in the gut affect IBD risk. Gut flora interact with multiple environmental factors, which may alter the composition or density of gut microbiota (*36*). It has been observed that IBD patients have lower overall gut bacteria diversity. However, certain intestinal bacteria species, such as adherent-invasive Escherichia coli, are overpopulated in CD patients compared to healthy individuals (*37*). In contrast, Firmicutes, as a potential beneficial species protecting against IBD, is less populated in the intestine of IBD patients (*38*). Because of the complexity of gut flora populations and their interactions with the host mucosa immune system, gene-sequencing technologies and bioinformatics tools are widely used to characterize gut microbiota composition change through IBD development (*39*). Further investigation needs to be done to identify whether such bacterial population alterations are causal factors to induce IBD.

Immune system and IBD. IBD is primarily characterized by immune cell over-activation induced by commensal bacteria and self-antigens in GI tract, making it an autoimmune disease (*40*). For example, intestine Mϕs may have lost tolerance to commensal bacteria and thus produce high level of TNF-α (*41*). This can further increase

Mϕ and T cell gut infiltration and thus induce a more robust immune response in the gut (*40*). Moreover, the pro-inflammatory response is not limited to local tissue but is also seen systemically. For example, IBD patients often have elevated serum TNF-α concentration compared to healthy population (*42*). The pathogenesis of two IBD sub-types are different regarding the dominant adaptive immune response. CD is correlated with an increase in Th1 and Th17 response, while UC is predominantly associated with elevated Th2 response (*43*).

1.2.3 The mechanisms of IBD development

The etiology and pathogenesis of IBD have been investigated for many years and current evidence suggests that mucosa microbiota alteration, mucosa barrier dysfunction and abnormal mucosa immune response promote IBD development (*44*). This process is also influenced by genetic alteration and environmental factors (*44*, *45*).

1.2.3.1 Mucosa barrier function and IBD

Intestinal epithelium functions as the first barrier of host defense, where it prevents invasive pathogens from entering the host tissue. It contains different mucosa components and intestinal cells with distinctive roles. The first protective layer is a physical mucus layer which prevents the direct colonization of gut microbiota on mucosa. Goblet cells are the main resource of mucus protein (*46*). IBD patients often have reduced goblet cell count or defected mucus production (*47*). The protective role of mucus has been further demonstrated in animal studies by showing that Muc-2 deficient mice (which lack mucus production) developed spontaneous experimental colitis in mice, but Muc-2 wild type ones did not (*48*). Within the intestine mucus layer, anti-microbial

peptide (AMP) produced by Paneth cells is another important compound to prevent bacteria infiltration (*49*). AMP is part of the innate immune mediators which kills a broad spectrum of bacteria in the intestinal lumen (*50*). Reduced AMP level has been seen in the intestinal mucus of IBD patients, which suggests the potential protective effect of AMP against IBD (*51*).

Intestinal epithelial cells connect to one another by forming tight junctions (TJ) which protect the gut epithelial monolayer integrity against luminal antigen infiltration (*52*). IBD patients often have "leaky gut", which demonstrates increased gut permeability with loosened cell-cell connection. This leaking phenotype may be caused by reduced TJ protein levels (e.g. ZO-1 and Claudin-2) or altered protein locations (e.g. E-cadherin), both of which are associated with IBD development (*53*). The current understanding of the initiating factors to mucosa barrier impairment remains unclear (*54*). Recent evidence has shown that pro-inflammatory cytokines (e.g. TNF-α) can increase gut permeability by altering TJ and inducing epithelial cell apoptosis, further initiating local immune response (*55*). Moreover, CD patients responding to anti-TNF-α treatment often had an improvement in gut epithelium integrity and permeability (*56*). This data indicates the importance of altered TJ functions on IBD initiation.

In addition to forming a physical protective barrier, intestinal epithelial cells are also involved in innate immune activity. Many epithelial cells have pattern recognition receptors expressed on their apical surface (e.g. TLR4), basal surface (e.g. TLR9) and cytoplasm (e.g. NOD2) (*57*). Toll-like receptor (TLR) expressed on epithelial cell apical surface induces immune tolerance. Under normal conditions, commensal bacteria will be recognized by TLR without inducing a pro-inflammatory response (*58*). As bacteria

infiltrate into the cell or reach the lamina propria, NOD2 in the cytoplasm and TLR on the basal side will recognize the antigens and initiate the innate immune response. NOD2 or TLR signaling will further activate the epithelial cell NF-κB signaling pathway and the invading foreign antigens will be cleared out (*58*).

1.2.3.2 Gut microflora and IBD

CD and UC frequently develop in the distal ileum and colon where large amounts of bacteria are located. This suggests that gut microbiota may be associated with IBD development (*59*). Studies with germ-free animals provid evidence that gut bacteria may be a causal effect on IBD. Germ-free mice failed to develop spontaneous colitis (*60*), and instead develop milder dextran sulfate sodium (DSS)-induced colitis compared to mice raised under normal conditions (*61*). In general, two potential mechanisms have been proposed to explain IBD initiation from gut microflora. One is persistent infection caused by gut pathogens, and the other is dysbiosis. The latter describes the shifted balance of beneficial and detrimental bacteria populations in the gut.

With overall high bacteria density in IBD patients' gut lumen, some potential pathogens are overpopulated which may contribute to IBD initiation. For example, Adherent-Invasive E. coli. (AIEC), also known as CD-associated E. coli, is highly populated in the ileum of 22% CD patients (*37*). The significance of AIEC pathogenic function was further strengthened in animal studies that transfection of AIEC in mice was sufficient to induce gut inflammation (*62*). AIEC has an extremely high capacity to colonize to mucosa, infect intestinal epithelial cells and initiate Mϕs mediated pro-inflammatory response (*63*). AIEC also is also present in relatively lower amounts among

healthy populations than in CD patients, suggesting that AIEC may be a conditional pathogen and more likely to cause disease in sensitive individuals (*64*).

Dysbiosis describes a condition of luminal microbial imbalance, which is another possible cause of IBD. By analyzing washed colonic biopsies from 305 IBD patients and 40 healthy individuals, Swidsinski et al. demonstrated that the overall density of mucosa bacteria in IBD patients was significantly increased compared to healthy controls (*59*). They also reported that the bacteria density in IBD patients was positively related with disease severity. Frank et al. used sensitive molecular-based sequencing analysis and further characterized the microbiota shift in IBD patients (*65*). Firmicutes (~60%), Bacteroidetes (~30%), Proteobacteria and Actinobacteria phyla are four major microbiota populations in a healthy gut. In IBD patients, a significant depletion of Firmicutes and Bacteroidetes was observed, whereas increased numbers of Proteobacteria and Actinobacteria were found. This evidence suggests that maintaining luminal bacteria balance may protect against human IBD.

1.2.3.3 Immune system and IBD

IBD and innate immune response. Mononuclear phagocytes, including Mϕs and dendritic cells (DC), are the most abundant cell types in the innate immune system and have central roles in maintaining intestinal mucosa homeostasis and in regulating immune response. Mucosa Mϕs have high phagocytic activity but do not migrate to lymph nodes, whereas DCs migrate to lymph nodes and prime naïve T cells. In intestine steady state, mucosa Mϕ is responsible for detecting and clearing out luminal bacteria. Unlike inflammatory Mϕs, mucosa Mϕs constantly produce high levels of IL-10 and low

levels of TNF-α, and their interaction with commensal bacteria in steady state does not induce pro-inflammatory cytokine production. Unlike in healthy individuals, Mϕs in CD and UC patients' mucosa may have lost their immune tolerance to luminal bacteria. The number of pro-inflammatory Mϕs ($CD14^+$) is often increased in IBD patients (*66*), leading to increased levels of Mϕ produced mediators in the gut, such as TNF-α, IL-12, IL-1β, and reactive oxygen and nitrogen species. Mϕs in inflamed gut are derived from blood monocytes and commonly classified into two categories, M1 and M2. This classification is based on their cell surface markers and biological functions (*67, 68*). M1 Mϕs are classically activated Mϕs induced by LPS and IFN-γ. They express high levels of major histocompatibility complex (MHC) II, produce large amounts of pro-inflammatory mediators (i.e. ROS, TNF-α, etc), and function through defending against mucosa infection and activating adaptive T cell response (*69*). On the other hand, M2 Mϕs are non-classically activated Mϕs polarized by IL-4 and IL-13(*70*). They express high levels of CD206 on cell surface and produce higher levels of growth factors (e.g TGF-β) and anti-inflammatory cytokines (e.g. IL-10). These M2 produced immune regulators facilitate mucosal healing and suppress the local immune response (*71*). M1 and M2 phenotypes are interchangeable and the balance between the two is highly relevant to experimental colitis severity (*72-74*). An increased M1 to M2 ratio has been observed during colitis progression and a reduced ratio was seen during recovery (*73, 74*). This implys that M2 Mϕ is important for mucosa healing by producing ant-inflammatory cytokines (e.g. IL-10) and growth factors (e.g. TGF-β). Delayed mucosa healing was observed in Mϕ specific TGF-βRII signaling deletion (CD68-dnTGFβRII) mice after DSS treatment (*75*). Moreover, adoptive peritoneal transfer of M2 Mϕ

eliminated Dinitrobenzene sulfonic acid (DNBS) induced experimental colitis severity in mice (*76*). This suggests the important role of M2 Mϕs on IBD recovery. In addition, clinical observation show that IBD patients who respond to anti-TNF-α treatment also have increased $CD206^+$ Mϕ (M2) number in intestine (*77*).

Similar to Mϕs, DCs also have two sub populations responsible for immune stimulating or immune regulating effects (*78*). Most of the work on DCs has been done in mice even though the DC population in mice and humans may not be identical. Mucosa DCs in mice express cell surface marker CD103 and CD11b (*78*). Under healthy conditions, mucosa DCs are hyporesponsive to commensal bacteria, and they are therefore considered as tolerogenic DCs. These cells produce IL-10 and functions by activating regulatory T cells and suppressing adaptive immune response. DCs loss of immune tolerance has been linked to increased risk of IBD (*79, 80*).

IBD and adaptive immune response. IBD patients also experience disrupted adaptive immune response (T and B cell differentiation and activation) in the intestine, which is directly regulated by innate immune cells. (*81, 82*). Early studies mainly focused on shifted Th1 and Th2 balance in IBD. Th1 lymphocytes, which are activated by M1 Mϕ and DC, are over-activated in patients with active CD. Activated Th1 cells produce high levels of TNF-α, IL-12 and IFN-γ which further activate Mϕs and induce cytotoxic effects (*82*). In contrast, UC patients are often diagnosed with Th2 cell overpopulation. Activated Th2 cells produce high levels of IL-4, IL-5, IL-13 and TGF-β, and further activate B cell induced humoral response (*83*). The antibody-dependent cell-mediated cytotoxicity (ADCC) induced by B cells may further cause prolonged tissue damage in UC.

An imbalance in the response of two more recently discovered cell types, Th17 and Treg, also contributes to IBD development (*84*). Both cell types are differentiated from Th0 and commonly regulated by TGF-β (*85*). In pro-inflammatory environment (high levels of IL-6 or IL-21), TGF-β favors the differentiation of Th17, while in steady state or anti-inflammatory environment (high levels of IL-10), it promotes Treg differentiation (*85*). Th17 is activated by IL-23, which is produced by activated M1 Mϕs and NK cells. Increased Th17 cell infiltration and Th17 related cytokines (e.g. IL-23 and IL-17A) have been observed in the inflamed mucosa of both CD and UC patients (*86*). This suggests that Th17 cell response may be associated with IBD pathogenesis. Indeed, GWAS studies showed strong association between Th17 cell related genes (e.g. IL-23R) and human IBD (*87*). However, animal studies created controversy regarding the role of Th17 cells in colitis. IL-17A gene deletion increased the disease severity of DSS-induced experimental colitis (*88*), whereas IL-17R knockout significantly protected against 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis (*89*). These results suggest that Th17 regulates IBD development in a much more complex fashion. As mentioned before, Th17 and Treg balance is important for gut homeostasis and mucosa response to environmental insults. Tregs play an important role in immune tolerance and suppression, which may reduce the risk of auto-immune response (*90*). Since IBD has long been considered as an auto-immune disease, the regulation of Treg on IBD has been extensively studied. The potential clinical relevance of Treg and IBD has recently been reviewed (*91*).

B cell mediated mucosa humoral immune response has also been associated with IBD pathogenesis. Increased numbers of infiltrating B cells (including plasma cells) were observed in the intestine of both CD and UC patients, and the phenotype of

immunoglobulin produced by plasma cells skewed from IgA to IgG in IBD (*92-94*). In normal mucosa, IgA is the main immunoglobulin subtype which keeps the gut homeostasis from three aspects. First, IgA complex binds invading pathogens and effluxes them to the lumen through pIgR (*95*). Second, IgA potentially suppresses neutrophils and Mϕs recruitment to the gut (*96*). Third, it reduces monocytes and Mϕs producing TNF-α (*97*). In acute infection, IgG is beneficial because it is a much more efficient form than IgA to enhance Mϕ phagocytic property and remove invading pathogens. However, IBD patients have sustained high IgG levels in the gut, which become deleterious due to IgG induced chronic inflammation and potential auto-immune response (*98, 99*). In CD, increased IgG is predominantly anti-E Coli, whereas in UC, the IgGs are mainly targeting colonic epithelial cells. In addition, overproduction of IgG can over-activate complement system and ADCC effect which may cause further tissue damage and delayed mucosa healing (*93, 100*). B cell mediated immune response in IBD is a fine and complex regulation and further research is required to explore the mechanisms.

1.2.3.4 Mucosal healing and IBD

Mucosa healing, which describes epithelium integrity and functionality recovery, has been merged into a crucial IBD therapeutic goal (*101, 102*). When steroid medicine was first used to treat UC, the clinicians observed that the treatment was more favorable in the patients who had both clinical and endoscopic remission compared to those who were clinically recovered only (*103*). Rapid mucosa healing was later observed in CD patients who responded to anti-TNF-α treatment (*104*). Collectively, these observations raised

research interest in the mucosa healing process which could influence the natural course of both UC and CD. Clinical mucosa healing is defined as the resolution of ulcer and erosion in IBD under colonoscopy (*105*). Mechanistically, mucosa healing is a complex biological procedure that depends on the communication between intestinal epithelial cells, immune cells and stroma cells (*106*).

Mucosa healing is a multi-step process, including epithelial restitution, proliferation and differentiation. Epithelial restitution is the earliest healing response which happens only minutes after mucosa surface injury (*107*). The epithelial cells residing adjacent to the wound depolarize, quickly migrate, and seal the open ulcer. Restitution is tightly regulated by growth factors and cytokines (*108, 109*). For example, TGF-α is a growth factor that induces epithelial cell migration by activating ERK-MAPK signaling pathway. Right after restitution, epithelial cells undergo rapid proliferation and crypt fission, resulting in increased cell and crypt numbers in the ulcer bed. Epithelial cell proliferation during healing is dependent on the activation of NF-κb and STAT3 signaling pathways. The activation is favored by certain local cytokine induction, such as IL-6 and IL-22 (*110*). Some other cytokines, such as TNF-α, would induce epithelial cell apoptosis instead, thereby delaying the healing process (*111*). The TLR family (such as TLR2, TLR4 and TLR9) and downstream signaling pathways may be beneficial during wound healing. For example, TLR2 expressed on colon epithelial cells can be activated by bacterial antigens, which will then induce goblet cells producing Trefoil factor 3 (TFF3) (*112*). TFF3 is an apoptotic compound and its deficiency is associated with delayed mucosa healing in colitis. This may be due to reduced epithelial proliferation or stem cell

survival rate (*113*). Following stem cell mediated colonocytes differentiation, the crypt structure and function will be recovered.

1.2.4 Current management and treatment strategies on IBD

Multiple treatment strategies have been developed to induce the remission of IBD, including modulating inflammatory mediators, lymphocytes migration and luminal bacteria population. One of the earliest anti-inflammatory medicines for IBD treatment is corticosteroids, which are effective in both UC and CD patients (*114*). Corticosteroids work quickly, are easy to use, and are not as expensive as other medicines. However, these drugs are only effective in a short term, and thus do not satisfy the role of sustaining IBD remission (*114*). Moreover, there are also toxicity concerns about this drug. Corticosteroids are a universal anti-inflammatory medicine that cause overall immune suppression, which increases the infection risk of IBD patients (*115, 116*). Therefore, corticosteroids need to be applied with other medicines to improve the treatment outcomes in IBD.

Anti-TNF-α antibody is also widely used to clinically treat IBD. The most common drugs are infliximab and adalimumab (*117, 118*). Both are effective for treating acute CD, but infliximab is more effective than adalimumab for treating UC (*117, 118*). The advantage of anti-TNF-α treatment is that the drug only targets one pathogenesis cytokine instead of the overall immune response, and as a result no side effects of anti-TNF-α treatment have been reported to increase infection or cancer risk (*119*). By combining infliximab and thiopurines, the short duration of remission is significantly improved compared to using infliximab alone (*120, 121*). The longer duration of infliximab may be because of its role in improving wound healing in the gut. These advantages expand the

usage of this drug in the clinic, but there are also limitations. First, the anti-TNF-α antibody is usually expensive (*122*). Second, about one third of IBD patients do not respond to this treatment, and even those who initially respond to it will build up tolerance and lose the treatment effect (*123*). This suggests that diagnosis and treatment should be personalized, and that alternative strategies are needed for IBD treatment.

Vedolizumab is a monoclonal antibody that specifically prevents lymphocytes adhesion and migration to the gut. It functions through blocking α4β7 integrin and ligand binding. The major advantage of this drug is that it is gut specific and the systemic immune toxicity is significantly reduced. Vedolizumab is a new IBD treatment medicine approved by the FDA in 2014, and has been used as an alternative treatment option for those that do not respond to anti-TNF-α treatment (*124*). Clinical studies showed that treatment of Vedolizumab to UC and CD twice (at week 0 and week 2) significantly improved remission by week 6 (*125*). Long term usage of the drug for a year resulted in significant IBD remission compared to placebo controls (*126*). A new drug specifically targeting β7 is being developed, with the goal of improving the drug specificity in gut mucosa only (*127*).

Since gut microbiota play a non-negligible role in IBD development, antibiotics and probiotics are used to control the total number and balance of bacteria. Antibiotics are used to treat active stage IBD especially when bacteria translocation is observed (*128*). A meta-analysis concluded the beneficial effect of antibiotics on IBD treatment by comparing 10 clinical trials using different antibiotics compounds in various populations with either active or quiescent IBD (*129*). Probiotics have also been used under inconsistent clinical conditions and thus the evidence of its efficacy on IBD treatment is

not as strong. It works effectively for UC treatment in some circumstances but has no beneficial effect on managing CD (*130*).

1.3 Overview of vitamin D

Vitamin D is a fat soluble pre-hormone traditionally known to regulate calcium homeostasis and maintain bone health. Vitamin D has two main forms named vitamin D_2 and vitamin D_3 (*131*). Vitamin D_2 is primarily found in plants, while vitamin D_3 is the dominant form in oil rich animal resource diet. Vitamin D_3 can also be synthesized in skin from 7-dehyrocholesterol under UV exposure (*132*). Vitamin D binds to vitamin D binding proteins and circulates in the blood, requiring two conversion steps to become active. First, vitamin D circulates to the liver and is converted to 25(OH)D by the enzyme 25 hydroxylase (*133*). 25(OH)D is a stable form of vitamin D, whose serum concentration is used as the biomarker to evaluate an individual's vitamin D status. The second step is to convert 25(OH)D to its active form, 1,25 dihydroxyvitamin D ($1,25(OH)_2D$) by 1α-hydroxylase (CYP27B1) (*134*). This enzyme is mainly present in kidney tubular epithelial cells, and also in extra renal tissues at a relatively low level (*135*). 24-hydroxylase (CYP24A1) is the enzyme that can convert excess levels of 25(OH)D and $1,25(OH)_2D$ in target tissue to their inactive forms, 24,25 dihydroxyvitamin D and 25 hydroxyvitamin D-26,23-lactone, and then to the water soluble form calcitroic acid to be excreted via urine (*134*).

The United States Institute of Medicine (IOM) defines vitamin D deficiency as serum 25(OH)D level below 30 nmol/L (12 ng/ml), and considers the level of 50 nmol/L (20 ng/ml) to be adequate (*136*). To reach adequate serum 25(OH)D level, the

Recommended Daily Allowance of Vitamin D is 600 IU for general population between age 1-70. However, this definition of vitamin D deficiency has been challenged by the United States Endocrine Society's Clinical Practice Guideline, which states that serum 25(OH)D level of 75 nmol/L (30 ng/ml) is the cut-off for adequacy (*132*). Based on the fact that more than half of the US population does not reach adequate vitamin D status, the recommended vitamin D intake should be 1500-2000 IU per day (*132*).

$1,25(OH)_2D$ as an active hormone, binds with vitamin D receptor (VDR) and regulates gene transcription. VDR is expressed in multiple tissues, including two cell populations that this review is focused on, intestine/colon epithelial cells and immune cells. When VDR binds with its ligand $1,25(OH)_2D$ in the cytoplasm, it further heterodimerizes with retinoid X receptor (RXR) and translocates to the nucleus. VDR-RXR dimer will bind to the vitamin D response element (VDRE) in the regulatory region of its target genes to enhance or inhibit transcriptional events (*137*).

1.4 Vitamin D and IBD in human and rodent models

Growing epidemiological evidence showed that the prevalence of vitamin D deficiency is associated with increased IBD risk, including both CD and UC. The potential causal effect of low vitamin D status and IBD development is partially demonstrated in clinical trials and further supported by animal studies.

1.4.1 Vitamin D and IBD epidemiology

Worldwide epidemiological observation has shown that people living at high latitudes or being exposed to limited sunlight have increased risk of IBD (*138, 139*). Specifically, IBD prevalence has been observed to follow this geographic distribution in

Northern Europe and North America (*140*). For example, a large population based observation study showed that the CD and UC incidence significantly increased in those who lived farther way from the equator in North America (*141*). In Europe, when those who originally lived near the equator migrated to countries in the north, the risk of UC and CD also increased (*142*). Since the majority of vitamin D can be synthesized from skin under sunlight exposure, the hypothesis is that low vitamin D status is associated with high IBD risk. Indeed, vitamin D deficiency is often diagnosed among IBD patients and with a seasonal pattern (*143*). For example, data showed that 18-50% of IBD patients have serum 25(OH)D levels lower than 20 ng/ml at the end of summer, whereas this deficiency can be seen in 50-68% of IBD patients in late winter (*144*). Low vitamin D status in winter also correlates with the highest IBD incidence in early spring throughout the year (*145, 146*). In addition, vitamin D status is also negatively related with IBD severity and outcomes. Data from a cross-sectional study showed that Crohn's Disease Activity Index (CDAI) is lower in those patients with higher serum 25(OH)D levels (*12*), while patients with lower 25(OH)D levels had greater IBD disease activity (*147*). In addition, IBD risk has also been associated with VDR polymorphisms, suggesting that vitamin D signaling is a determinant factor of human IBD development (*148-150*).

1.4.2 Vitamin D supplementation and IBD: clinical trials

As described above, observation studies showed that vitamin D status is negatively correlated with IBD severity and outcomes, and hence clinical trials are necessary to test the role of vitamin D supplementation on IBD treatment. So far, no clinical trial has been done to test the effect of vitamin D on ulcerative colitis, while three clinical studies have been done test CD patients' disease severity with vitamin D supplementation.

The first open label trial included 35 patients with CD, treated with either 0.25 μg $1,25(OH)_2D$ or 1000 IU 25(OH)D. CD patients with $1,25(OH)_2D$ supplementation but not 25(OH)D had significantly reduced CDAI and serum C-reactive protein levels in the short term (*151*). Jørgensen et al. reported the first multicenter, randomized, double-blind clinical trial to evaluate the effect of vitamin D supplementation on CD therapy and disease remission. 108 CD patients were recruited and given either 1200 IU/day vitamin D_3 or placebo, and followed up for 12 months. 3 months vitamin D_3 supplementation significantly increased serum 25(OH)D status, and 12 months treatment reduced the disease relapse rate (p=0.06) (*29*). In another open label trial, 18 CD patients with low to moderate CDAI were recruited and given up to 5000 IU Vitamin D_3 per day for 24 weeks. Vitamin D_3 supplementation with the reported dose did not induce any adverse effect or toxicity, and significantly increased serum 25(OH)D level and reduced CDAI score (*152*).

1.4.3 Vitamin D and mouse models of IBD

Rodent models have been used to understand the potential mechanisms of vitamin D mediated protection from IBD. Mouse IBD models are mostly used, which include chemical induced models (e.g. DSS and TNBS), bacteria induced infection models, T cell transfer models, and spontaneous IBD models (e.g. IL-10 knockout (KO)) (*153*). Vitamin D mediated protection against experimental colitis was tested by 1) feeding mice diets with sufficient and deficient vitamin D content, 2) applying $1,25(OH)_2D$ or its analogs as colitis treatment strategy, and 3) using global VDR KO, conditional VDR KO and VDR transgenic models to block or recover vitamin D induced transcriptional regulation (*154-156*). So far, multiple evidence has shown that vitamin D is a protective factor against

experimental colitis, and that the protection is mediated through regulating the gut barrier function, immune function and gut microbiota population.

Intestinal mucosa, containing mainly intestinal epithelial cells and lamina propria immune cells, is the biological barrier of the digestive system. Mucosa epithelial damage and chronic inflammation cause GI tract dysfunction and systemic symptoms (*157*). Vitamin D has been proposed to have protective roles in maintaining gut barrier integrity. As early as 2008, Kong et al. showed that VDR KO mice developed more severe colon ulceration after DSS-induced colitis. These mice also had reduced transepithelial electrical resistance (TER) compared to controls, indicating increased gut barrier permeability (*158*). In C. Rodentium induced mouse colitis model, low vitamin D intake resulted in more severe bacteria infiltration in cecum and secondary lymphoid tissues (*159*). Intragastric delivery of $1,25(OH)_2D$ rapidly recovered DSS-induced colon damage, paracellular permeability and bacteria translocation, suggesting that the vitamin D-mediated mucosa barrier protection was through colon epithelial cells (*160*). To further specify the role of VDR in intestinal epithelial cells, Liu et al. used the villin-human VDR transgenic model and found that global VDR deletion induced severe gut leaking in TNBS induced colitis can be corrected when intestinal VDR was recovered (*161*). Vitamin D regulates gut permeability by upregulating TJ gene expression levels (*160, 162*), maintaining appropriate TJ protein locations (*163*), and inhibiting colon epithelial cell apoptosis (*164-166*). Details will be discussed in the later section of this review. Vitamin D also protects Paneth cell survival, differentiation and its anti-microbial function (*167*). Intestine specific VDR KO mice were more susceptible to DSS-induced colitis and developed disordered, depleted and diffused Paneth cells during DSS

treatment. Meanwhile, reduced ATG16L1 expression level in Paneth cells of intestine VDR KO mice was observed, suggesting impaired autophagy function of the cells (*167*). Anti-microbial gene Ang4 was also shown to be a potential Vitamin D regulating target because mice fed low vitamin D diet had reduced Ang4 mRNA and protein levels after DSS treatment (*168*).

Vitamin D functions as an adaptive immune modulator during experimental colitis and thereby protects against disease development. VDR KO mice developed more severe colitis induced by DSS compared to wild type controls, and symptoms included elevated intestine inflammation and pro-inflammatory cytokine production in colonic tissue (*154*). This suggests that increased sensitivity of VDR KO mice to DSS is potentially through altered immune response. Previous research mainly focused on the impact of vitamin D signaling on T cell response. First, vitamin D alleviates experimental colitis by suppressing Th1 while inducing Th2 cell response (*155, 169-171*). For example, $1,25(OH)_2D$ oral treatment significantly increased anti-inflammatory cytokine IL-10 (Th2 and Treg cytokine) levels while decreased pro-inflammatory cytokine IFN-γ (Th1 cytokine) in DSS-induced colitis model (*154*). A similar effect was also found in T-cell mediated TNBS-induced colitis model, that $1,25(OH)_2D$ analog calcitriol or ZK156979 treated mice had reduced Th1 and induced Th2 and Treg response in the colon (*155, 171*). IL-10 is a Th2/Treg produced anti-inflammatory cytokine which inhibits colitis development, while deletion of IL-10 gene induces spontaneous colitis in mice. VDR/IL-10 double KO had increased small intestine and colon structure disruption compared to IL-10 single gene deletion, together with induced IL-1β, IL-2, IFN-γ and TNF-α concentration in local gut (*172*). This observation is consistent with an early report that

$1,25(OH)_2D$ supplement reduced small intestine inflammation and overall disease activity in IL-10 KO mice (*173*). Second, vitamin D also regulates Th17 cell response in colitis. IL-17 is significantly increased in VDR KO IBD mice with increased severity of tissue damage (*174*). This is consistent with increased Th17 cell numbers in damaged mucosa compared to controls (*174*). In the RAG KO model with depleted T and B cells, VDR KO T cell adoptive transfer increased colitis severity with increased IL-17 producing cell numbers both in the colon and in circulation (*174*). This suggests that VDR signaling is important for regulating Th17 cell differentiation and activation. In addition, VDR KO mice had reduced intraepithelial lymphocytes CD8 $\alpha\alpha^+$ T cell numbers in gut mucosa, which has an anti-bacterial effect in the gut (*175*). This may partially explain the increased sensitivity of VDR KO mice to salmonella induced colitis (*176*).

Gut bacteria are also associated with colitis pathogenesis, and vitamin D is proposed to inhibit colitis development by maintaining the balance of gut microbiota populations. Data showed that Cyp27b1 KO and VDR KO mice developed more severe DSS-induced colitis, which was associated with increased detrimental bacteria species (Bacteroidetes and Proteobacteria phyla) and reduced beneficial bacteria species (Firmicutes and Deferribacteres phyla) in the feces (*162*). Similarly, the altered gut microbiota were also observed in vitamin D deficiency mice which were more susceptible to developing experimental colitis (*163, 177*)(*159*). This regulation on microbiota is partially through vitamin D signaling in intestine epithelial cells. Inactivating VDR in intestinal epithelial cells is sufficient to alter gut microbiota populations, such as reducing the number of Butyrate producing bacteria, Butyrivibrio (*167*). Probiotics can also regulate VDR

expression in intestinal epithelial cells; they then induce epithelial cell anti-microbial activity and suppress pathogen induced colitis (*178*).

1.5 Vitamin D and immune response

The earliest evidence of vitamin D's immune regulation function was reviewed through its usage on treating mycobacterial diseases (e.g. tuberculosis (TB)) 70 years ago, even though the mechanism was not clear at that time (*179, 180*). For a long time, the importance of vitamin D and its effect on the immune system was widely recognized because VDR was found in multiple immune cells types. Active form of vitamin D, $1,25(OH)_2D$ was found to bind to transcription factor VDR in T cells, B cells, monocytes/Mϕs, DCs and neutrophils, suggesting that immune cells may directly respond to circulating $1,25(OH)_2D$ (*181-185*). Meanwhile, other studies discovered that human Mϕs also express CYP27B1, which encodes the enzyme to convert 25(OH)D to its active form $1,25(OH)_2D$ (*186*). This highlights the potential that $1,25(OH)_2D$ can regulate immune cell function in an autocrine manner. The following session, further discusses the molecular mechanisms of vitamin D mediated innate and adaptive immune response regulation.

1.5.1 Vitamin D and innate immune cells

Two major types of innate immune cells, Mϕ and DCs, are both vitamin D responding cells and their biology is tightly associated with IBD. Mechanistically, vitamin D regulates monocyte /Mϕ anti-microbial effects, proliferation and differentiation, migration and immune regulation. Vitamin D also influences DC antigen

presentation and maturation. Details of these regulation mechanisms will be discussed below.

Vitamin D and its anti-microbial effects on monocyte/Mϕ. Vitamin D's anti-microbial effects were first observed from its usage in treating TB, and then emphasized in a large-scale in-silico and microarray study that two anti-bacteria genes, cathelcidin (cAMP) and β-defensin 2 (DEFB4) were upregulated by $1,25(OH)_2D$ (*187*). DR3 consensus VDREs were found in the promoter regions of both genes, suggesting that the two genes are direct vitamin D regulating targets (*187*). However, it has later been proved that only cAMP can be transcriptionally regulated by $1,25(OH)_2D$ in monocytes, whereas DEFB4 expression can only be indirectly regulated by VDR when pro-inflammatory transcription factor NF-κb is co-activated (*187*). It was later discovered that NOD2 gene transcription was directly regulated by vitamin D, which resulted in NF-κb activation and increased DEFB4 expression (*188*). When monocytes were co-treated with $1,25(OH)_2D$ and NOD2 ligand (muramyl dipeptide), NF-κb dependent DEFB4 enhancement was observed (*188*). Genetic variants of NOD2 are associated with increased CD risk (*189*). For example, G908R, R702W and L1007 frameshift deletion of NOD2 have been linked to the development of CD, which is due to insufficient activation of bacterial killing capacity (*19*). Therefore, vitamin D has anti-microbial effects and protects against human IBD.

Vitamin D and monocyte/Mϕ proliferation and differentiation. In addition to its anti-microbial effect, $1,25(OH)_2D$ also suppresses human PBMC proliferation. Sito et al. showed that $1,25(OH)_2D$ or its analogues (EB 1089, KH 1060) significantly reduced the proliferation of PBMCs in CD patients (*190*). Vitamin D also induces monocyte-Mϕ cell

lineage differentiation. Early studies showed that 1,25$(OH)_2$D alone induced the differentiation of bone marrow derived Mϕ (BMDM) in both mice and humans based on observation of morphology change (*191*). In the presence of M-CSF or L929 culture medium, 1,25$(OH)_2$D co-treatment further induced mouse BMDM cell surface mannose receptor levels, while dietary vitamin D deficiency in mice caused reduced F4/80 and MAC-2 expression levels on primary cultured BMDM (*192, 193*). This suggests that vitamin D signaling influences Mϕ maturation from bone marrow cells. Interestingly, bone marrow cells from VDR KO mice developed similar amounts of Mϕ colonies compared to those from mice with normal VDR, with or without M-CSF (*191*). This implies that vitamin D is not necessary for BMDM development when M-CSF is sufficient. Moreover, 1,25$(OH)_2$D was proposed to induce monocytes differentiation into Mϕs in vitro. 1,25$(OH)_2$D alone significantly increased human PBMC MAX1 and MAX3 expression, induced cell adherence, and stimulated cell inflammatory response to LPS (*194, 195*). Similarly, 1,25$(OH)_2$D also induced CD14 and CD11b expression on THP-1 cells, suggesting induced Mϕ differentiation. (*196-199*). However, those cells derived from THP-1 with increased Mϕ cell surface markers failed to become adherent, thus they may not be matured Mϕs (*196, 199*). Collectively, the mechanism of vitamin D regulated Mϕ differentiation requires further investigation to explain the discrepancy between different experimental models. Due to practical reasons, whether vitamin D can induce bone marrow myeloid precursors to develop into monocytes remains unclear. Monocytes are differentiated from bone marrow hematopoietic stem cells, which is induced by M-CSF and through PU.1 transcription factor (*200*). M-CSF can be produced by multiple cell types including osteoblasts and human PBMCs, and the M-CSF expression can be

elevated by 1,25$(OH)_2$D treatment in culture (*201, 202*). Similar effects had been seen in phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) induced THP-1 and HL-60 cell lines that 1,25$(OH)_2$D induced the M-CSF production from cells (*202*). This suggests that vitamin D signaling may regulate monocyte differentiation indirectly through inducing M-CSF related signaling pathways. M-CSF binds to M-CSFR and activates multiple connected signaling pathways. By activating Src kinase, both PI3K/Akt and PLC-γ /PKC/ERK pathways can then be activated (*200*). These events will in turn activate multiple transcriptional factors (e.g. C/EBPα, c-Jun, NF-κb etc.) and eventually upregulate PU.1 expression and function (*200*). Among these pathway components, 1,25$(OH)_2$D has been shown to increase PI3K activity in THP-1 cells, and VDR is necessary to TCR induced PLC-γ1 expression in human T cells (*198, 203*). These results indicate that vitamin D may be a potential monocyte lineage differentiation modifier by regulating M-CSF initiated signaling pathways.

Vitamin D and monocyte/ Mϕ migration. Systemic inflammation induces bone marrow monocyte production and emigration, thus increased circulating monocyte numbers (*204*). Monocyte emigration from bone marrow is dependent on the MCP-1/CCR2 pathway (*204*). Monocytes express high levels of CCR2 on the cell surface. CCR2 deletion on monocytes caused monocyte accumulation in the bone marrow after LPS injection in mice, and thus induced impaired systemic immune defense (*204*). Vitamin D was proposed to have anti-migration effects partially through its suppression of CCR2 expression on monocytes/ Mϕs (*205, 206*). MCP-1 is the chemokine ligand of CCR2 which can be produced by multiple cell types. Among those, monocytes and Mϕs are the major source of MCP-1 (*207*). Serum MCP-1 is mainly produced by circulating

monocytes under steady state, which ensures newly differentiated monocytes emigrating into the circulation and replenish blood monocyte population. Data showed that mouse serum MCP-1 level was significantly elevated when fed a western diet containing low vitamin D and calcium, and the chemokine level was reduced in mice with sufficient vitamin D and calcium intake (*208*). Serum MCP-1 concentration is positively correlated with circulating $CD11b^{+}$ monocyte numbers in mice (*209*) and is upregulated in both DSS and TNBS induced experimental colitis model in mice (*210, 211*). However, whether low vitamin D signaling induces high serum MCP-1 levels or increased monocyte numbers in blood circulation remains unknown. In vitro, LPS induced MCP-1 production by THP-1 cells and THP-1 induced Mϕs, and this pro-inflammatory effect was significantly suppressed by $1,25(OH)_2D$ pre-treatment in both groups of cells (*212*). This suggests that $1,25(OH)_2D$ may directly regulate blood monocyte MCP-1 expression and thus induce bone marrow monocyte emigration.

Vitamin D signaling may also inhibit monocyte migration to local tissue mediated by MCP-1. Colonic MCP-1 expression was increased in mice with DSS induced colitis, and further elevated in mice with intestinal VDR deletion (*206, 211*). In Azoxymethane (AOM)/DSS induced colon cancer mouse model, VDR deletion caused increased tumor burden in colon with significantly increased MCP-1 expression level in colon tumor (*213*). This suggests that vitamin D may also function on colon epithelial cells and reduce their production of MCP-1, and therefore reduce monocyte migration into the colon.

Vitamin D and its immune suppressive function on monocyte/Mϕ. Monocytes and Mϕs express VDR and the VDR activation can induce a significant immunosuppressive effect on those cells (*214*). In human monocytes, 1,25 D treatment

inhibited the expression of MHC class II antigens, including human leukocyte antigen (HLA)-DR, HLA-DP, and HLA-DQ, all of which contribute to antigen presentation (*215, 216*). 1,25$(OH)_2$D suppresses a variety of pro-inflammatory cytokines in human monocyte/Mϕs cell lines mechanistically through suppressing AP-1 and NF-κB–mediated pro-inflammatory pathways (*217-219*). The similar 1,25$(OH)_2$D mediated immune-suppressive effect was also seen in mouse primary cell cultures. For example, 1,25$(OH)_2$D treatment significantly suppressed LPS induced IL-1β and TNF-α expression in mouse peripheral blood mononuclear cells (PBMC) and BMDM (*220, 221*), and IFN-γ induced oxidative burst and host defense mediators, including CCL5, CXCL9, CXCL10, FcγRI, and TLR2 in murine BMDMs (*222*). Since all of these immune-suppressive effects were observed in activated monocytes/Mϕs, but not in resting cells, the mechanism of 1,25$(OH)_2$D induced anti-inflammatory response in monocyte/Mϕ is proposed to be a feedback loop regulation (*223*).

Vitamin D and mature Mϕ polarization. Vitamin D modulates Mϕ activation and polarization which then influences colitis related epithelial damage and healing. As has been mentioned before, Mϕ polarization and activation are tightly related to colitis development and recovery (*73, 74, 224*). NF-κB and STAT6 are two transcription factors regulating M1 and M2 polarization respectively. LPS activated Mϕ NF-κB transcriptional activity is inhibited by 1,25$(OH)_2$D through increased IκB expression (*225*), while IL-4 activated STAT6 pathway might be induced by vitamin D (*226-228*). Evidence showed that 1,25$(OH)_2$D treatment increased STAT6 expression and enhanced Th2 polarization in the multiple sclerosis (EAE) model (*227*), and THP-1 human monocyte cell line was polarized to M2- like cells by 1,25$(OH)_2$D (*228*). However, it is not clear whether STAT6

is a $1,25(OH)_2D$ direct target or whether the $1,25(OH)_2D$ promotes M2 polarization through this pathway. A recent study showed that $1,25(OH)_2D$ can also induce M1 to M2 class switch. M1 Mϕs were polarized from RAW264.7 cell line by high glucose treatment, characterized by high expression levels of TNF-α and IL-12(*229*). $1,25(OH)_2D$ treatment significantly reduced the M1 cytokine expression levels and increased M2 markers Arg1 and IL-10, potentially through upregulating PPARγ signaling pathway (*229*).

Vitamin D and DC regulation. DCs are professional antigen presenting cells (APCs) expressing both VDR and cyp27b1 (*230*). Even though DCs are developed from the same hematopoietic lineage as monocytes/Mϕs, their response to vitamin D appears to be maturation and antigen presentation regulation (*230*). While DCs are differentiating into mature APCs, they express increased levels of cyp27b1, but reduced levels of VDR. One explanation is that immature DCs with high VDR expression will respond to $1,25(OH)_2D$ produced by mature DCs and undergo differentiation (*231*). This allows some but not all DCs to become mature APC and therefore prevents an over-activated T cell response that may cause further immune complications (*231*). VDR and cyp27b1 KO mice developed abnormal lymphatic response presented by increased mature DC numbers but reduced DCs trafficking (*232*) (*233*) (*234*). This emphasized the important role of vitamin D in suppressing DC maturation and promoting DC migration. Similar to Mϕ phenotypes mentioned above, DCs also have two different subtypes, myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). mDCs have higher efficiency to active naive T cells while pDCs function as immune suppressive cells to attenuate T cell activation (*80, 235*). Even though mDCs and pDCs express similar levels of VDR, mDCs are

$1,25(OH)_2D$'s preferable regulating targets in vitro (*236*). This $1,25(OH)_2D$ mediated regulation on mDCs may further influence the downstream adaptive immune response.

1.5.2 Vitamin D and adaptive immune response

Adaptive immune cells, including T and B cells, also express VDR and are potential vitamin D targets. T cells develop in the thymus and then leave after maturation. Pre-mature T cells in the thymus express VDR, but the expression level will be dramatically reduced once they emigrate into blood circulation. Activated T cells in secondary lymphoid tissue will have their VDR expression recovered, suggesting that activated T cells, not naïve T cells are vitamin D targets. Data showed that $1,25(OH)_2D$ inhibits T cell expansion induced by APCs in vitro, likely because $1,25(OH)_2D$ inhibits T cell proliferating cycle from early to late G1 phase.

Vitamin D also regulates T cell differentiation and activation. T cells are involved in adaptive immune response as a diverse population, which can be further classified as helper T cells, regulatory T cells and cytotoxic T cells. Among those, the effects of vitamin D on helper T cells have been best characterized. Naïve T helper cells (Th0) require APCs to be activated, and differentiated into Th1 (induced by IL-12, IFN-r), Th2 (induced by IL-4, 5, 10) or Th17 (induced by IL-23) based on cytokine profiles in the microenvironment (*237*). In addition to suppressing effector T cell proliferation as mentioned before, vitamin D can also regulates T cell activation and cytokine production. Multiple studies showed that $1,25(OH)_2D$ and its analogs reduced Th1 cytokine (e.g. IFN-γ) production in vitro, (*238-240*), and a similar effect was also observed in disease models in vivo. For example, vitamin D successfully prevented Th1 induced spontaneous autoimmune disease development (e.g. SLE) in mice by inhibiting Th1 induced cytokine

and IgG2a production (*239*). In addition, Boonstra et al. have demonstrated that vitamin D also affects T cell differentiation by inhibiting Th1 and favoring Th2 (increased production of IL-4, IL-5, IL-10) when the naïve T cells were co-cultured with either splenic APC or the combination of anti-CD3 and anti-CD28 (*241*). Mechanistically, vitamin D increased IL-4 induced Th2 transcriptional factors expression, including c-maf and GATA-3 (*241*). $1,25(OH)_2D$ can also suppress Th17 cytokine IL-17A expression in both human and mouse T cells in culture (*242*). In the mouse EAE model (disease model of human multiple sclerosis), $1,25(OH)_2D$ injection attenuated disease severity by suppressing IL-17A producing cell activity in the blood and central nervous system (*242*).

In addition to regulating Th cell response, vitamin D also promotes $CD4^+CD25^+$Treg induced anti-inflammatory and immune tolerogenic function (Barrat et al., 2002). The mechanism of how vitamin D regulates Treg is still controversial. $1,25(OH)_2D$ was first demonstrated as an indirect regulator on Treg through suppressing DC maturation in vitro (*236*). Subsequently, however, $1,25(OH)_2D$ was described to directly influence CTLA-4 and Foxp3 positive Treg generation (*243*), and a functional VDR binding site VDRE was found in the human Treg Foxp3 promoter region (*244*). Not only the active form $1,25(OH)_2D$ but also the pro-hormone 25(OH)D were found to influence Treg development. Jeffery et al. showed that 25(OH)D, especially non-DBP bound free 25(OH)D, has the ability to increase Treg generation through DCs (*245*). The importance of vitamin D effect on Treg has also been illustrated in several in vivo studies. For example, $1,25(OH)_2D$ administration was used to treat chronic kidney disease by increasing circulating Treg numbers in humans (*246*). In addition, the serum 25(OH)D

concentration is significantly correlated with Treg activity in human MS (*247, 248*). Similarly in mice, $1,25(OH)_2D$ and its analog increased Treg cell numbers in the draining lymph nodes (*249, 250*).

$CD8^+$ cytotoxic T cell is another potential vitamin D regulating target due to the high VDR and cyp27b1 expression levels (*251, 252*). $CD8^+$ cytotoxic T cells are responsible for defending against virus infection and cancer, and the effect of vitamin D on these cells has recently been reviewed (*253*). A special group of $CD8^+$ T cells, $CD8\alpha\alpha^+$ T cells, which do not have cytotoxic effects, may have an immune suppressive function in the GI tract (*254*). Data showed that global VDR KO mice developed less $CD8\alpha\alpha^+$ T cells in the intestine which may associated with increased colitis severity (*255*). It may be caused by reduced chemokine CCR9 expression and impaired T cell homing in VDR KO mice (*255*).

Besides T cell response regulation, some early evidence showed that vitamin D can also influence B cell proliferation and development (*256, 257*). The mechanism was initially proposed as an indirect regulation through helper T cells. It was then demonstrated that $1,25(OH)_2D$ can directly inhibit plasma cell differentiation and memory B cell class switch (*238, 258*). More recent reports showed that $1,25(OH)_2D$ also regulates B cell cytokine and chemokine production (e.g. IL-10 and CCR10), suggesting additional roles besides regulating immunoglobulin secretion (*259, 260*).

1.6 Vitamin D and epithelial barrier function

1.6.1 Vitamin D maintains gut permeability

The integrity of the gut barrier is the key to keep intestine homeostasis and prevent chronic inflammatory diseases caused by "leaky gut". Vitamin D has been proposed to maintain gut permeability through regulating TJ, adherens junctions and epithelial cells apoptosis.

1.6.1.1 Vitamin D and TJ in the gut

TJ is a special paracellular structure made of a complex of proteins. It seals the gaps between epithelial cells and forms a barrier to prevent luminal molecules from invading into deep tissue. The protein complex includes three parts: cytoskeletal proteins, cytoplasmic proteins and membrane proteins. The major structural and functional component of TJ proteins are Claundins, Occludins and "zonula occludens" (ZO) protein family (*261*). In multiple experimental colitis studies, VDR deletion increased gut permeability and $1,25(OH)_2D$ treatment recovered the gut integrity. For example, Zhao et al. observed that DSS-induced gut cellular permeability and bacteria translocation was associated with impaired TJ, and $1,25(OH)_2D$ intragastric administration significantly recovered ZO-1, Occludin and Claudin-1 gene expression and protein levels in intestinal mucosa (*160*). Vitamin D signaling also regulates the cellular location of TJ proteins during infection. Assa et al. showed that E-coli infected Caco-2 cells had reduced TER and paracellular permeability, potentially caused by internalization of TJ proteins. $1,25(OH)_2D$ treatment prevented the pathogen induced redistribution of ZO-1 and Claudin-1 thus rescued the single cell layer integrity in vitro (*163*). This effect was

independently observed by Kong et al. that deleting VDR in Caco-2 cells resulted in the internalization of TJ proteins (*158*). Christakos et al. also showed that 1,25$(OH)_2$D upregulated intestinal claudin-2 level (*262*), and the mechanism was later proved by Zhang et al. that claudin-2 is a direct vitamin D regulating target (*263*). The Zhang group found a VDRE site within the claudin-2 promoter region and proved that VDR directly upregulated claudin-2 expression in both transcriptional and translational levels (*263*).

1.6.1.2 Vitamin D and adherens junctions

Adherens junction (AJ) proteins also form a complex to connect adjacent epithelial cells in the intestine. Contrary to TJs, AJs are located at the basal part of epithelial cells. AJ protein complex includes transmembrane proteins (e.g. E-cadherin) and intracellular compartments (e.g. β-catenin). Cyp27b1 KO and VDR KO mice colon epithelial cells expressed lower levels of E-cadherin after DSS treatment, indicating E-cadherin may be a regulating target of vitamin D to protect against colitis (*162*). In vitro evidence consistently showed that 1,25$(OH)_2$D treatment increased E-cadherin expression in Caco-2 cell culture and enhanced reconstitution after injury (*158*). Vitamin D regulated β-catenin activities have been mainly reported under colon cancer circumstances. Colon cancer cells have β-catenin nuclear translocation, and 1,25$(OH)_2$D treatment prevents β-catenin from entering the nucleus (*264*). This effect has only been seen in human SW480 cells with VDR expression but not those cell lines lacking VDR (e.g. SW620) (*264*). In vivo, Apc min mice with VDR deletion resulted in increased β-catenin nuclear translocation and colon tumor size (*265*). However, whether the regulating is applicable to normal intestinal cells or relevant to IBD is still unclear.

1.6.1.3 Vitamin D and intestinal epithelial apoptosis

Vitamin D induces colon cancer cell apoptosis, while inhibiting colon epithelial cell death during mucosal healing. Previous studies on colon cancer cell lines showed that vitamin D can directly upregulate bcl-2 and induce apoptosis (*266*). Indirectly, vitamin D can inhibit Mϕ activation and IL-1β expression, therefore sensitizing colon cancer cell response to TRAIL induced apoptosis (*267*). However, these mechanisms are restricted to non-cancer colon epithelium. Under normal conditions, vitamin D protects colon epithelial cell survival by reducing oxidative stress and DNA damage (*268*). Liu et al. were later the first to report the anti-apoptosis role of vitamin D on colon epithelial cells during experimental colitis (*164*). VDR KO mice were more susceptible to TNBS-induced colitis and their colon epithelial cells apoptosis rate (TUNEL staining) was significantly higher than controls. They also showed that recovering intestinal VDR reduced the apoptosis rate in intestine epithelial cells by suppressing the pro-apoptotic molecule PUMA (*164*).

1.6.2 Vitamin D regulates Paneth cells function

About 3% of the mouse and human genomes are potentially regulated by vitamin D, which indicates that vitamin D may be involved in the development of multiple diseases (*269*). Specifically in IBD, two Paneth cell related anti-microbial genes, AMP and β-defensin, have been proved to be direct vitamin D regulating targets. In addition, IBD risk is linked to dysregulated autophagy gene ATG16L1 which is also expressed in Paneth cells and regulated by VDR target gene NOD2 (*270*). With this information, Wu et al. conducted a study to investigate the direct effect of vitamin D signaling on Paneth cell autophagy during experimental colitis (*167*). They found that intestine epithelial cell

VDR KO increased colitis severity in mice with reduced ATG16L1 expression levels and impaired autophagy function in Paneth cells. This regulation was independently reproduced by knocking down VDR in culture. More importantly, they found a VDRE site in the promoter region of mouse ATG16L1, and confirmed that it is a functional VDR binding site using CHIP assay (*167*). This study filled the gap of vitamin D regulation on Paneth cell autophagy function and its relationship with IBD development.

1.7 Research gaps

As has been discussed before, vitamin D mediated protection against IBD has been seen in both human and animal models and the potential mechanisms are summarized in figure 1.1. In mouse models, vitamin D signaling significantly reduced the severity of experimental colitis at the acute stage. However, human IBD is often diagnosed at late stage and the disease duration strongly affects IBD patients' life quality and causes further health issues. Therefore, the goal of human IBD management is to alleviate IBD clinical symptoms with significant intestine mucosa healing. So far, the question of whether or how vitamin D signaling influences mucosa healing is still to be answered. More importantly, VDR is widely expressed in multiple cell types in the colon, and has independent effects on colon epithelial cells and immune cells. Since they both contribute to colitis development and recovery, it is important to know which type of cells plays a more important role in vitamin D mediated protection against IBD. In the following chapters, I will first introduce an animal model I developed, which was later used to investigate colon epithelial cell VDR function in colitis induced mucosa healing (Chapter 2). I will then report the experiments I conducted to compare the impact of vitamin D

between colon epithelial cells and immune cells during experimental colitis (Chapter 3). Based on my own research data, I further investigated the regulatory effect of vitamin D on one type of immune cells, Mϕs (Chapter 4). At the end of the thesis, I will discuss future research directions (Chapter 5).

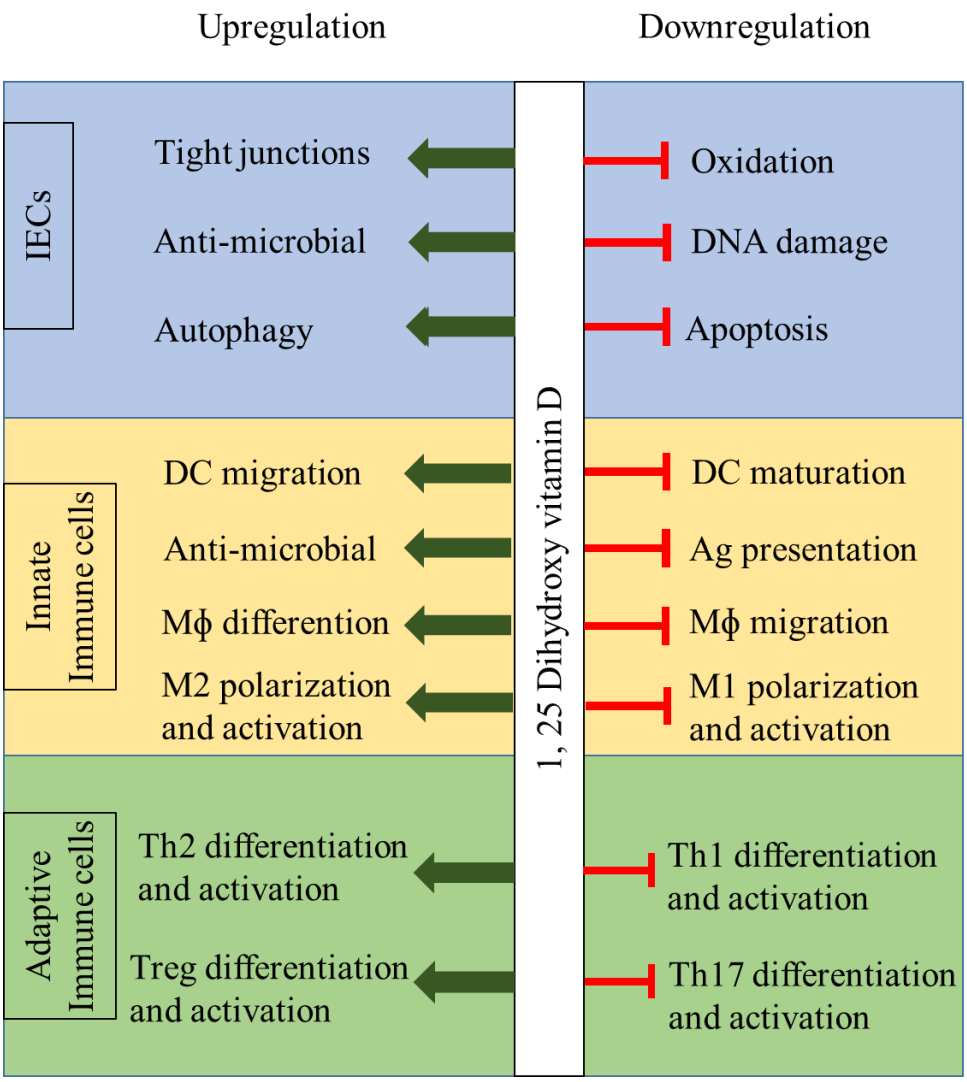

Figure 1.1 Summary of the Potential Vitamin D Mediated Cellular Events Relevant to IBD.

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CHAPTER 2. AN INDUCIBLE, LARGE-INTESTINE-SPECIFIC TRANSGENIC MOUSE MODEL FOR COLITIS AND COLITIS-INDUCED COLON CANCER RESEARCH

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2.1 Abstract

Background Animal models are an important tool to understand intestinal biology. Our lab previously generated C57BL/6-Tg(*Car1*-cre)5Flt transgenic mice (CAC) with large intestine specific Cre recombinase (Cre) expression as a model to study colon health.

Aims To expand the utility of the CAC mouse model by determining the impact of chemically induced colitis on CAC transgene expression.

Methods CAC mice were crossed to Rosa reporter mice (*Rosa26R*$^{flox/flox}$) with a lox-STOP-lox signal controlling β-galactosidase (βgal) expression and then further crossed with Apc$^{CKO/CKO}$ mice in some experiments to delete Apc alleles (Apc$^{\Delta 580}$) Initially, 8-wk-old CAC$^{Tg/WT}$;*Rosa26R*$^{flox/WT}$;*Apc*$^{\Delta 580/WT}$ mice were treated with dextran sulfate sodium (DSS) in drinking water (5 d, 0, 0.65%, 1.35%, or 2.0%). Colon tissue damage and βgal labelling were analyzed 10-d after stopping DSS. Next, 8-wk-old CAC$^{Tg/WT}$;Rosa26R$^{flox/flox}$ mice were treated with 0 or 1.35% DSS, and colonic β-gal labeling was assessed at 30-d post DSS treatment. Finally, 10-wk-old CAC$^{Tg/WT}$;*Apc*$^{\Delta 580/WT}$ mice were treated with DSS (0 or 2%) for 5 d and colonic tumors were analyzed at 20-wk.

Results CAC$^{Tg/WT}$;*Rosa26R*$^{flox/WT}$;*Apc*$^{\Delta 580/WT}$ mice had a DSS dose-dependent increase in colon epithelial damage that correlated with increased epithelial β-gal labeling at 10-d (r^2 = 0.9, β = 0.75). The β-gal labeling in CAC$^{Tg/WT}$;Rosa26R$^{flox/flox}$ mice colon remained high at 30-

d, especially in the crypts of the healed ulcer. DSS also increased colon tumor incidence and multiplicity in $CAC^{Tg/WT}$;$Apc^{\Delta 580/WT}$ mice.

2.2 Introduction

Colorectal cancer is a multi-factorial disease and its development can be influenced by genetics, diet, inflammation, environment and other factors (*1-6*). Unfortunately, the etiology of colorectal cancer remains unclear, thus barriers exist to develop approaches to prevent and treat the disease (*7-9*). Controlled animal studies are an important approach to understand colorectal cancer biology and to test promising strategies for disease prevention and treatment. As such, an optimal animal model of colorectal cancer should capture the pathological changes, molecular mechanisms, and clinical symptoms presented in the human disease. In addition, the cancer effect should be limited to colon to avoid the confounding effect of cancer in other tissues.

Many animal models have been developed for colorectal cancer research, and they have been reviewed in several recent comprehensive reviews (*10-12*). Our lab previously generated a transgenic mouse model for colorectal cancer research with large intestine-specific Cre recombinase (Cre) transgene expression, i.e. the C57BL/6-Tg(Car1-cre)5Flt or CAC mouse (*13*). In colon epithelial cells of CAC mice, Cre is expressed in approximately 10% of the epithelium. Here we report that Cre expression in colon epithelial cells of CAC mice can be increased during dextran sulfate sodium (DSS) induced colitis. In addition, our data suggest that these recombination events induce a persistent effect that can be used to study genes involved in the epithelial response to colitis or in colitis-associated colon cancer.

2.3 Materials and Methods

2.3.1 Animals

C57BL/6-Tg(*Car1*-cre)5Flt mice were created by our group (*13*) and maintained as heterozygotes ($CAC^{Tg/WT}$). The *Rosa26R* strain, B6.129S4-*Gt(ROSA)26Sortm1Sor*/J, was obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained as homozygotes ($ROSA^{flox/flox}$). *Rosa26R* mice contain a STOP sequence that is flanked by LoxP sites upstream from the *E. coli* β-galactosidase (βgal) gene within a gene trap of the *Rosa26* allele (*14*). The Apc^{CKO} mouse carrying a floxed exon 14 of the *Apc* gene ($Apc^{CKO/CKO}$) was obtained from the Mouse Models of Human Cancer Consortium (National Cancer Institute, Frederick, MD). In cells expressing Cre, exon 14 is removed resulting in a truncated 580 amino acid long protein produced from one ($Apc^{\Delta 580/WT}$) or both ($Apc^{\Delta 580/\Delta 580}$) Apc alleles. Genotypes were determined by standard PCR methods as previously described for the CAC (*13*), *Rosa26R* (*14*), and floxed *Apc* alleles (*13*). Each strain of mouse was maintained on a C57BL/6J background. Mice were bred to create experimental animals with specific combinations of genotypes. All mouse breeding colonies were housed individually, given a standard chow diet and water *ad libitum*, and exposed to a 12 hours light/12 hours dark cycle. The experiments were conducted with approval from the Purdue University Animal Care and Use Committee.

2.3.2 Experimental Design

Experiment 1: $CAC^{Tg/WT}$;$Rosa26R^{flox/flox}$ mice were crossed to $Apc^{CKO/CKO}$ mice to generate $CAC^{Tg/WT}$;$Rosa26R^{flox/WT}$;$Apc^{\Delta 580/WT}$ mice. Experimental mice were weaned at 3 weeks of age and fed the AIN93G diet (*15*). Mice were randomly assigned into one of four dextran sulfate sodium (DSS) treatment groups (0%, 0.65%, 1.35% and 2%, n=16 per group, 8 male and 8 female). DSS (MW = 36,000-50,000 Da, MP Biomedicals, LLC, Solon, OH) was

diluted in deionized water (w/v) to the appropriate concentrations. At 8 weeks of age, mice received the DSS solution in place of water *ad libitum* for 5 consecutive days. Afterwards, DSS was replaced with deionized water and this was provided until the day of sacrifice. Half of the mice in each group were sacrificed 2 days after ending the DSS treatment and half were sacrificed 10 days after ending DSS treatment. All mice were fasted overnight before harvest. At necropsy, the proximal and distal colon were prepared as Swiss rolls and examined for β-galactosidase levels by immunohistochemistry.

Experiment 2: 24 $CAC^{Tg/WT}$;$Rosa26R^{flox/flox}$ mice (12 female and 12 male) were weaned at 3 weeks of age and fed AIN93G diet. At 8 weeks of age, 16 mice (8 male and 8 female) were treated with 1.35% DSS for 5 days and the other 8 mice (4 male and 4 female) were used as vehicle controls (0% DSS). Half of the DSS treated mice and all the vehicle control mice were sacrificed 10 days after completing the DSS treatment. The other mice in the DSS treatment group were sacrificed 30 days after completing the DSS treatment. All mice were fasted overnight before harvest. At necropsy, the proximal and distal colon were prepared as Swiss rolls and examined for β-galactosidase levels by immunohistochemistry. Small intestine, kidney, spleen, liver and lung were also collected for detection of β-galactosidase enzymatic activity.

Experiment 3: 13 $CAC^{Tg/WT}$;$Rosa26R^{flox/WT}$;$Apc^{\Delta 580/\Delta 580}$ mice with two recombined Apc alleles were generated by crossing $CAC^{Tg/WT}$;$Rosa26R^{flox/WT}$;$Apc^{\Delta 580/WT}$ mice with $Apc^{CKO/CKO}$ mice. Mice were sacrificed at 4 weeks of age after an overnight fast. At necropsy, the proximal and distal colon were prepared as Swiss rolls and examined for β-galactosidase levels by immunohistochemistry.

Experiment 4: 81 CAC$^{Tg/WT}$;*Apc*$^{\Delta 580/WT}$ mice were generated by crossing CAC$^{Tg/WT}$ mice with *Apc*$^{CKO/CKO}$ mice. CAC$^{Tg/WT}$;*Apc*$^{\Delta 580/WT}$ pups were weaned at 3 weeks of age and fed an AIN93G diet. CAC$^{Tg/WT}$;*Apc*$^{\Delta 580/WT}$ mice were treated with either 2% DSS (n=38) or deionized water (n=43) at 10 weeks of age for 5 days, then the DSS was replaced with water. Mice were sacrificed at 20 weeks of age after an overnight fast. The colon was removed, opened longitudinally, and cleaned with PBS. Digital images were collected and analyzed to determine the number, location, and size of each tumor.

2.3.3 Detection of β-galactosidase Activity in Organs

Tissues were dissected from the mice, rinsed briefly in cold PBS, and fixed for 1-2 hours in ice cold 4% paraformaldehyde. After rinsing the tissue for 15 minutes in PBS, tissues were incubated for 4 hours in 1 mg/mL 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal; Invitrogen; B-1690) solution (pH 7.6) at 37°C as previously described (*13*). In tissues expressing βgal (indicative of Cre-mediated recombination of the *Rosa26R* locus), the enzyme converted the X-gal substrate into a dark blue crystalline precipitate that is visible upon gross examination.

2.3.4 Tissue Preparation and Immunohistochemical Detection of β-galactosidase

The colon was opened longitudinally, rinsed with cold PBS, and transected at its midpoint to separate the proximal and distal colon. Each section was split longitudinally and Swiss-rolled (*16*), resulting in 2 rolls of proximal colon and 2 rolls of distal colon per mouse. Tissues were immersion fixed for 48-72 hours in 10% neutral buffered formalin at 4°C before being transferred to 70% ethanol. Tissues were processed and embedded in paraffin within 7 days of collection at the Purdue Histology and Phenotyping Laboratory**.**

Four micron tissue sections were cut, de-paraffinized, and rehydrated through xylene, ethanol, and water by standard methods. For antigen retrieval, slides were submerged in 0.01 mol/L sodium citrate (pH 6.0) and heated to 96°C for 20 minutes in a laboratory microwave (PELCO, Edmond, OK). Immunohistochemistry was performed on a Dako Autostainer (Dako, Denmark). Slides were incubated with 0.03% hydrogen peroxide (5 minutes) and serum-free protein block (30 minutes; Dako; Denmark), followed by a 120 minute incubation with rabbit polyclonal anti–β-galactosidase (β gal) antibody (1:400 dilution; Novus; NB100-65209). Antigen binding was detected with a horseradish peroxidase–labeled anti-rabbit IgG polymer (Vector laboratories; MP-7410) and 3, 3'-diaminobenzidine (DAB; Vector laboratories; SK-4100) as the chromogen. Slides were counterstained with Harris hematoxylin (EK Industries, Juliet, IL). The digital images were collected with an Aperio ScanScope slide scanner (Leica Technologies, Buffalo Grove, IL) at 20X magnification.

2.3.5 Image Analysis

Mucosal transgene expression was determined by quantifying the percent βgal positive pixels after immunohistochemistry. The colonic mucosa was differentiated from the tunica muscularis using the Genie histology pattern recognition software (Aperio, Leica Biosystem) trained at 5X magnification and 1000 iterations to a mean training accuracy of 96%. A positive pixel count algorithm was then used to analyze DAB labeling within the mucosa. This analysis was done by a board certified veterinary pathologist (RLJ) and confirmed independently (by FW).

The area of βgal positive crypts was determined by manually measuring the length of the mucosal surface containing βgal labeled crypts. βgal positive crypts were included only if the entire crypt axis, from base to lumen, was within the plane of section or if the crypt base could

be seen immediately adjacent to the muscularis mucosa. The percentage of area represented by βgal positive crypts was calculated by dividing the length of the βgal positive area by the total length of the mucosa surface.

The damaged tissue surface area included the areas of ulceration and areas undergoing active healing. Ulceration was defined as sections of the colon mucosa devoid of crypts. This phenotype is obvious at day 2 after DSS termination and increased with DSS dose. Tissue healing included restitution (a single layer of epithelial cells covering an ulcer bed), crypt fission, and crypt regeneration - two events that repopulate the organized crypt structure within an ulcer. These phenotypes were observed at day 10 after DSS termination and continued through day 30 after DSS termination. The percentage of damaged surface was defined as the length of damaged mucosa divided by the total length of the mucosal surface.

2.3.6 Statistical Analysis

Statistical analysis was performed with SAS Enterprise 5.1 software (SAS Institute, Cary, NC). All data were reported as the mean ± standard error of the mean (SEM). Normality of the data was determined by evaluation of its histogram and a Shapiro-Wilk normality test ($p>0.05$). All of the data were normally distributed. Statistical comparisons were made by two-tailed student's t-tests, one-way ANOVA or Chi-square test as appropriate. Following one-way ANOVA, the Bonferroni multiple comparison test or planned orthogonal contrasts were used to compare groups. Differences were considered significant at $p \leq 0.05$ for all the statistical comparisons.

2.4 Results

2.4.1 Cre-mediated recombination is increased by DSS treatment in CAC$^{Tg/WT}$;*Rosa26R$^{flox/WT}$; Apc$^{\Delta 580/WT}$* mice.

Our group has previously reported that CAC mice have recombination of the Rosa allele that is restricted to the large intestine epithelial cells (*13*). In our experiments, β-gal labeling is used as an indicator of Cre-mediated transgene recombination. In experiment 1, we examined the proximal and distal colon of CAC$^{Tg/WT}$;Rosa26R$^{flox/WT}$;Apc$^{\Delta 580/WT}$ mice without DSS treatment (Fig 2.1A-D). The proximal colon under the basal, non-DSS condition had significantly more β-gal positive cells than the distal colon (Fig. 2.1E, p=0.0023). However, β-gal labeling in proximal colon was mostly superficial, while entire crypts were labeled in distal colon (Fig. 2.1B, D). As a result, 5-fold more β-gal positive crypts were observed in the distal colon than the proximal colon, (Fig. 2.1F, p=0.0011).

Ten days after DSS treatment β-gal labeling was significantly increased at all three DSS doses (Fig. 2.2A, vehicle vs DSS, Proximal colon: p<0.0001; Distal colon: p=0.0007). In both the proximal and distal colon, a significant increase in β-gal labeling was observed as more DSS was used, although the labeling plateaued in the 2% DSS treatment group (Fig. 2.2A). In addition, the increased β-gal labeling was present from the surface to the crypt base in both colonic segments (Fig. 2.3). The impact of DSS treatment on β-gal activity was also examined in the small intestine, liver, spleen, lung and kidney. As reported previously (*13*), β-gal activity was not observed in the small intestine, even after DSS induction (data not shown). Both liver and lung had low level baseline transgene recombination but this was not increased by DSS treatment. Kidney had non-specific β-gal activity in non-transgenic mice but this did not

increase in either the CAC mouse or the CAC mouse treated with DSS. In the spleen there was no transgene expression either at baseline or after DSS treatment (Fig. 2.4).

As expected, the percentage of damaged epithelial surface was higher with increasing DSS dose in both proximal and distal colon (Fig. 2.2B). In addition, there was a direct, positive correlation between epithelial damage and β-gal expression in both colonic segments ($r^2 \geq 0.9$, slope = 0.75, Fig. 2.2C). Also, regardless of the DSS dose, approximately 60% of the abnormal crypts in the areas of epithelial damage were β-gal positive (data not shown). This indicates that Cre-mediated recombination of the LacZ locus has occurred in the epithelium that expanded to heal the DSS induced ulcers.

2.4.2 Increased β-gal expression resulting from DSS induced colon damage is sustained after tissue recovery.

In normal colon mucosa, the crypts are well organized and separated by very little lamina propria (Fig 2.5A, B). 10 days after DSS treatment, the ulcerated mucosa was undergoing active healing, characterized by epithelial restitution, crypt fission, and crypt regeneration (Fig 2.5C, D). By 30 days after DSS treatment, the ulcerated mucosa was filled with normal-appearing crypts that were separated by slightly increased amounts of lamina propria (Fig 2.5E, F). As we observed in the first study, the total percentage of β-gal positive crypts was significantly higher in the DSS treatment groups than the vehicle controls at 10-day post DSS (proximal colon in Fig. 2.6A, $p<0.0001$, distal colon in Fig. 2.6D, $p=0.0024$) and greater than 60% of the crypts in the healing ulcer were β-gal positive (Fig. 2.6B, E). This increased β-gal staining remained 30 days after termination of DSS treatment (Fig. 2.5, Fig. 2.6, and Fig. 2.7). In fact, the percentage of β-gal positive crypts in the healing/healed ulcer was higher at 30-day

post DSS than 10-day post DSS (e.g. 75.2±4.4 % vs 57.9±6.3 % in proximal colon). The DSS treatment group also had significantly more β-gal positive crypts in areas of normal-appearing crypts that were adjacent to healing mucosa (Fig. 2.6C, F, Proximal colon: $p<0.0001$; Distal colon: $p=0.0001$). This indicated that the transgene expression induced by DSS was sustained in the tissue after recovery of the damaged epithelium.

2.4.3 DSS induced transgene recombination increases tumor formation.

We first examined whether β-gal staining occurs coincident with adenomatous phenotypes that result from deletion of two floxed Apc alleles. In $CAC^{Tg/WT}$;$Rosa26R^{flox/WT}$;$Apc^{\Delta 580/\Delta 580}$ mice, 30% of the distal colon exhibited an adenomatous phenotype consistent with recombination of both Apc alleles (i.e. increased cytoplasmic basophilia, increased nuclear to cytoplasmic ratio, loss of nuclear basal polarity, disruption of crypt architecture). While only 2.4% of crypts in normal mucosa were β-gal positive (Fig. 2.8A), within the adenomatous regions 88.8±1.5% of the epithelial cells were positive for β-gal (Fig. 2.8B). The co-occurrence of β-gal labeling (indicative of recombination at the ROSA26R locus) and the adenomatous phenotype (indicative of recombination of both floxed Apc alleles) suggests that Cre mediated recombination of floxed alleles is very efficient.

Since DSS treatment induces Cre-mediated recombination in crypts, we hypothesized that DSS would increase tumor incidence in $CAC^{Tg/WT}$;$Apc^{\Delta 580/WT}$ mice by expanding the colon-specific inactivation of a single Apc allele, coupled with the well-established tumor-promoting effects of inflammation (*17*). In the vehicle treated group, 18.6% of the mice had a colonic tumor and the tumors were all in the distal colon (Table 2.1, 1.25 per mouse). In contrast, DSS treatment significantly increased the percentage of mice with colonic tumors to 78.9% (DSS

vs Vehicle, $p<0.0001$) and this included the appearance of tumors in the proximal colon in 34% of mice. The number of colonic tumors developed per mouse was also significantly higher in the DSS treated mice compared to the vehicle controls (4.20 vs 1.25 per mouse, $p<0.0001$). The DSS treatment resulted in twice as many mice with tumors in the distal colon compared to the proximal colon (Table 2.1, 74% vs 34%, respectively) and twice as many tumors in the distal versus the proximal colon (Table 2.1, 3.71 vs 1.47 per mouse, respectively). Thus the CAC mediated recombination of Apc alleles induced by DSS along with the pro-inflammatory environment within DSS-induced ulcers was a potent promoter of colon cancer.

2.5 Discussion

Our results expand the utility of the CAC mouse model by demonstrating that recombination of floxed alleles driven by the Cre recombinase transgene is increased during DSS-colitis. This effect was restricted to large intestinal epithelial cells, was tightly correlated with DSS-induced epithelial damage, was visible during the healing of ulcers, and persisted in crypts within the healed epithelium. However, since DSS causes colon epithelial damage that is accompanied by local inflammation, it is not clear whether induction of transgene activity is a result of the epithelial damage, healing, inflammation, or the factors combined.

While the mechanism for this effect is not known, two lines of evidence suggest that the colonic stem cell has been affected. First, recombination is evident in epithelial cells from the luminal surface to the crypt base where colon stem cells reside, and second, the effect is persistent in crypts and lasts after the damaged epithelium has healed. $Lgr5^+$ cells have been identified as rapid turnover intestinal stem cells (*18*) that maintain the intestinal epithelium (*19*). However, $Lgr5^+$ stem cells are absent from the DSS damaged areas of the distal colon

(*20*). Other putative intestinal stem cells with moderate proliferative capacity have been identified including cells marked by Tert, Bmi1, Hopx, and Lrig1$^+$ cells. These stem cells may interconvert to maintain the intestinal epithelium, especially under stress, e.g. as seen when Bmi1$^+$ cells serve as the stem cell reservoir in the small intestine when Lgr5$^+$ cells are deleted (*21, 22*). Although Bmi1$^+$ cells are not present in the colon (*21*), Lrig1$^+$ cells are found in there (*23*). We hypothesize that CAC transgene expression may be activated in Lrig1$^+$ cells during healing and that recombined floxed alleles are retained in Lrig1+ cells which then pass them to new Lgr5$^+$ cells that repopulate the epithelium after ulcer healing. Further experiments examining transgene expression in Lrig1$^+$ and Lgr5$^+$ cells before, during, and after DSS induced colitis need to be done to test the hypothesis.

In addition to the CAC mouse we developed and used in these experiments (*13*), several other mouse models exist for colon research and each have advantages and disadvantages (*11*). A 12.4 kb region of the mouse villin promoter drives Cre recombinase expression uniformly throughout the epithelial cells of the intestine (*24*). However, villin-Cre transgene expression extends into the small intestine (*24*) and this can confound interpretation of events occurring in the colon. An improvement on the villin-Cre mouse was accomplished by using a 9 kb villin promoter to drive a CreER^{T2} transgene that is tamoxifen inducible and which can cause gene deletions that are spatially and temporally controlled. However, 60 days after treating villin-CreER^{T2} mice with tamoxifen, the persistence of recombination in small intestine was seen in 80% of the epithelium while in the colon it was just 40% (*25*). Moreover, villin promoter driven Cre or Cre-ER^{T2} transgene expression level is lower in the crypt base where stem cells are found (*24, 25*) and this suggests that Cre activity in the colon stem cell compartment may not be sufficient for optimal recombination.

Deletion of floxed alleles directly in intestinal stem cells can be accomplished using the Lgr5 promoter-CreER^{T2} mouse (*26*) and the Lrig1 promoter-CreER^{T2} mouse (*23*). Unfortunately, inducible Cre-ER^{T2} transgene activity is very low in the large intestine of Lgr5 promoter-CreER^{T2} mice (*27*) and so, like the villin promoter models, the Lgr5 promoter-CreER^{T2} mouse is best for inducing small intestinal cancer. The Lrig1 promoter-CreER^{T2} mouse forms multiple adenomas in the distal colon when it is used to delete an Apc allele; however, many tumors are also seen in the small intestine (*23*).

An improvement on these models is the CDX2 promoter-Cre mouse whose transgene expression is seen only in cecum, colon and distal ileum of adult mice. When it is used to delete a floxed Apc allele, tumors form in the ileum and distal colon (*28*). Unfortunately, transient expression of the CDX2-Cre transgene during embryonic development causes recombination of floxed alleles throughout the distal half of the body. This could reduce the utility of the CDX2-Cre model for many genes whose functions are present outside the colon. A tamoxifen-inducible version of this model, CDX2-CreER^{T2}, avoids the problem of embryonic transgene recombination (*27*). However, the induced transgene activity is much higher in proximal colon than the distal colon so its utility may be limited.

Our original CAC model (*13*) has a major advantage over the other models just described -intestinal transgene expression is specific to the large intestine. This makes the model useful to study the role of specific genes in basic colonic biology and in cancer. A weakness of the CAC model is that CAC transgene expression affects just a small portion of the colonic epithelium and extends to the crypt base mostly in the distal colon. Here we report a major advance for the CAC mouse – that Cre-mediated recombination is induced during DSS-mediated epithelial damage in the colon. The effect of DSS-induced colitis on CAC transgene

activity occurs to a comparable degree in both the proximal and distal colon. The DSS/CAC model also has the advantage that the increase in transgene activation is directly related to the dose of DSS used but that increase does not involve the entire mucosa. For example, we found that only 40-50% of the epithelial surface in the distal colon became transgene positive after treating mice with 2% DSS for 5 days. Thus, in this model both normal tissue (without transgene expression) and tissue with recombined alleles will be adjacent to one another. This will reduce the biological variation caused by intra-animal differences by providing an internal control for paired analysis of treatment effects.

An obvious use of the DSS/CAC model is to delete cancer-relevant genes and assess their impact on inflammation-induced colon cancer. As a preliminary test of this application, the DSS/CAC model was used to delete Apc gene alleles in the context of DSS-induced colitis. We found that in CAC mice with one inactivated Apc allele, DSS increased colonic tumor incidence 4-fold (to 78.9%) and tumor number per mouse 3-fold (to 4.5 colonic tumors per mouse). While this revealed the utility of our model, it also revealed a segment-specific risk for colon cancer. Despite the fact that DSS-induces recombination of floxed alleles equally in both proximal and distal colon, tumors formed predominantly in the distal colon. This suggests that the different environmental influences interact with inflammation and Apc allele loss to affect cellular transformation and tumor development in the proximal and distal colon.

Several other applications for the new DSS/CAC model could also benefit the colon cancer or IBD research community. First, since the CAC transgene is activated in epithelial cells during active colitis, our model could provide insight into the epithelial role of specific genes during active inflammatory bowel disease and during the epithelial healing response. For example, several novel genes have been proposed to influence colitis and colitis-induced

colon cancer through their impact on mucosal healing (e.g. NLRP6, TLR9, JAK3, FAK) (*29-32*). However, most of those genes are expressed in multiple cell types within the colon inflammatory microenvironment (e.g. NLRP6 is expressed in epithelial cells as well as macrophages, T cells, and B cells (*33*)). Because of this, the results from studies with global gene deletion models are confounded but the DSS/CAC model will allow one to overcome this problem and target the gene disruption directly to the site of ulceration and epithelial healing.

Second, our model may allow researchers to examine the importance of specific genes in field cancerization (*34-36*). This concept suggests that the cancer development starts in relatively large areas of tissue due to early pro-tumorigenic alternations. These mutations may not be sufficient to cause cancer but they predispose the tissue to further cancer-causing mutations. The molecular alterations that create a field or push a field towards cancer can be induced by micro-environmental triggers like colonic inflammation. For example, multiple bouts of tissue damage and repair in IBD patients increases colon cancer risk (*37*). In patients with Ulcerative Colitis who have developed neoplasia, TP53 or KRAS mutations can be detected in both the neoplastic area and non-dysplastic crypts (*38*). This suggests that the mutated cells had populated a large field of mucosa prior tumor formation. Our DSS/CAC model can be used to generate a wide spread field cells with specific gene mutations in the colon.

In summary, we observed a novel phenotype for the CAC model - that the Cre transgene mediated recombination is induced following DSS treatment. This opens the opportunity to study gene function at the time of colitis development as well as after the recovery of colitis. We believe the DSS/CAC model is an efficient, reliable model for research on the epithelial cell biology of colitis and inflammation-induced colon cancer. This will make the CAC/DSS

model a powerful tool to study the mechanisms by which inflammation alters the colonic epithelium (including cellular transformation) as well as to assess interventions to minimize the impact of inflammation on colonic diseases.

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Table 2.1 DSS Increases Tumor Incidence in $CAC^{Tg/WT}$;$Apc^{\Delta 580/WT}$ Mice

	Vehicle				DSS			
	n	# Tumors	Incidence (%)	# per mouse[a]	n	# Tumors	Incidence (%)	# per mouse[a]
Any Tumor	9	12	20.9	1.33±0.24	37	186	97.4*	5.03±0.56#
Cecum	2	2	4.7	1.00±0.00	30	60	81.6*	2.00±0.32#
Proximal colon	0	0	0.0	0.00	15	22	34.2*	1.47±0.29#
Distal colon	8	10	18.6	1.25±0.25	28	104	73.7*	3.71±0.35#
Proximal + Distal	8	10	18.6	1.25±0.25	30	126	78.9*	4.20±0.40#
No Tumor	34	0	79.1		1	0	2.6	
Total Mice	43				38			

Notes: Significantly different from the vehicle group (* Chi-square test, $p<0.05$; # Student's T-tests, $p<0.05$).

[a] Average number of tumors per tumor-bearing mouse

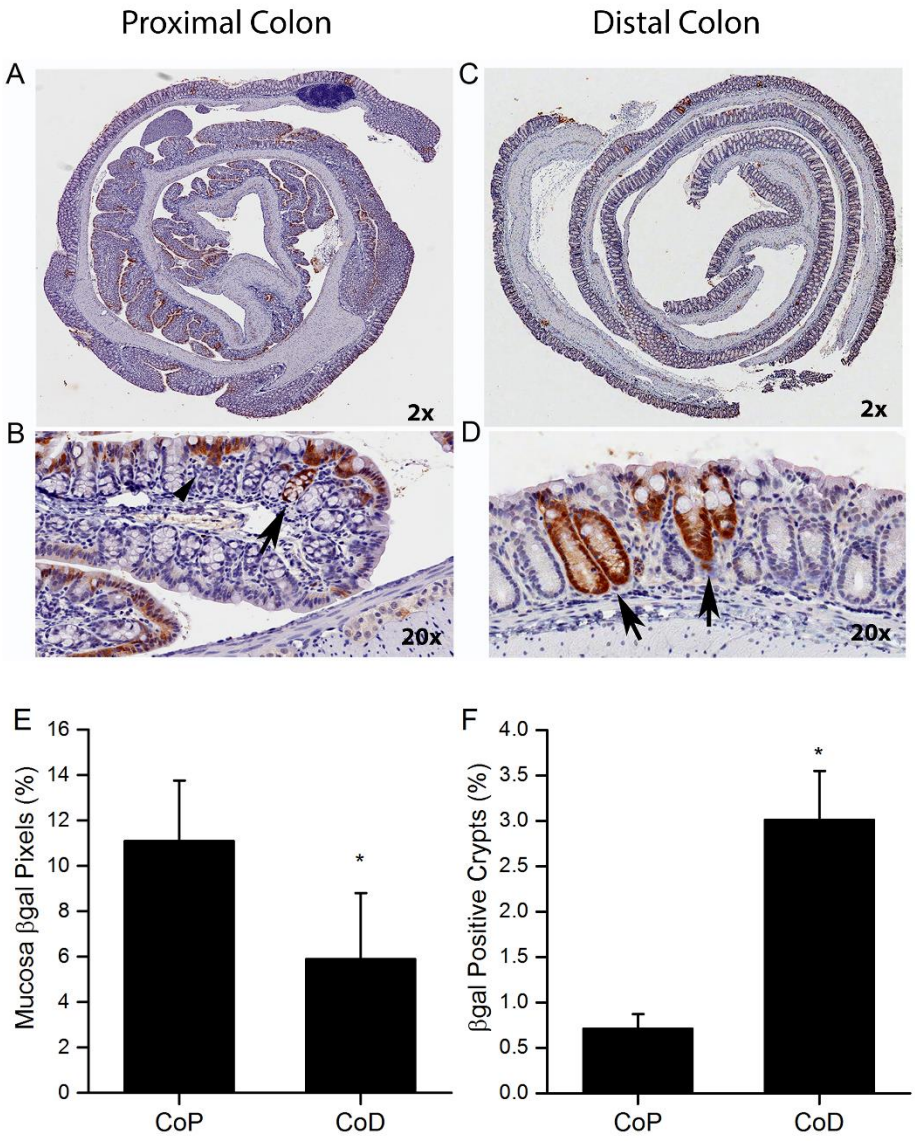

Figure 2.1 Baseline βgal expression patterns are different between proximal and distal colon in CAC$^{Tg/WT}$;*Rosa26R*$^{flox/WT}$;*Apc*$^{\Delta 580/WT}$ mice.Colons from 10 weeks old mice were prepared as Swiss rolls, processed, and stained for β-gal expression by immunohistochemistry. Representative images were generated using an Aperio ScanScope digital slide scanner and are presented for proximal colon (A, B) and distal colon (C, D), The top pictures are at 2X magnification and the bottom pictures are at 20X magnification. (B) β-gal labeling in proximal colon Swiss rolls was predominantly superficial (arrow head) with occasional labeling to the crypt base (arrow). (D) β-gal labeling in distal colon Swiss rolls was seen from the crypt base to the luminal surface (arrow). (E) Total mucosal β-gal positive pixels and (F) β-gal positive crypts were quantified. The bars represent the means ± SEM of 8 animals for data from the proximal colon and distal colon. * $p < 0.05$ vs CoP.

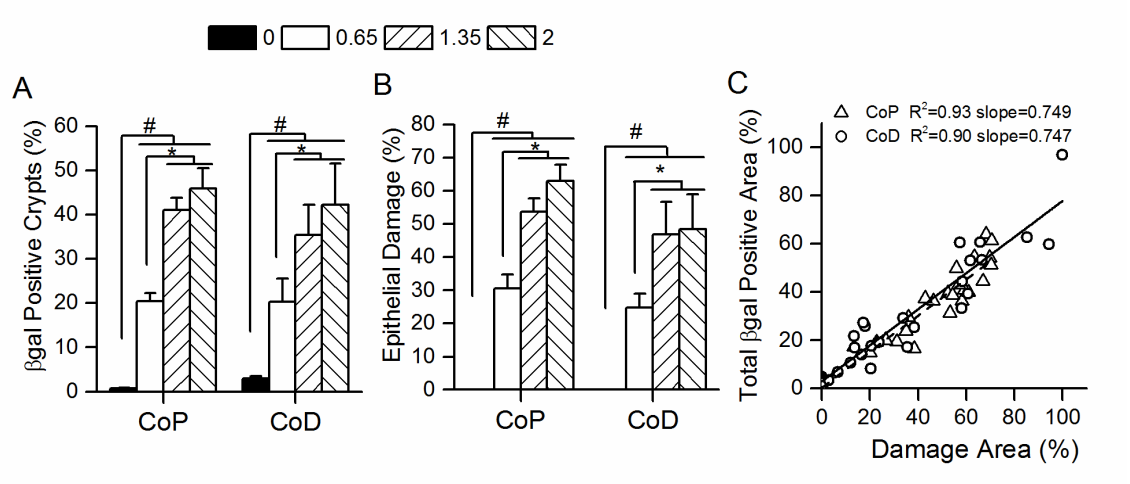

Figure 2.2 Colon β-gal expression is positively correlated with epithelial damage in CAC$^{Tg/WT}$;*Rosa26R*$^{flox/WT}$;*Apc*$^{\Delta 580/WT}$ mice following DSS treatment. Mice were given increasing amounts of DSS for 5 days then examined histologically 10 days later. (A) The percentage of β-gal positive crypts in the proximal or distal colon. (B) The percentage of damaged epithelium (includes colon epithelial ulceration, restitution, crypt fission and other abnormal crypt structures observed during the healing process). Bars represent the mean ± SEM of 8 animals per group. Planned orthogonal comparisons were performed between no DSS vs the DSS treatment groups (# $p<0.05$), the low DSS vs high DSS groups (* $p<0.05$), and the two high DSS groups (not significant). (C) The relationship between the percentage epithelial damage and β-gal positive area in colon. The regression line for the proximal colon is shown as a dashed line. The regression line for the distal colon is shown as a solid line.

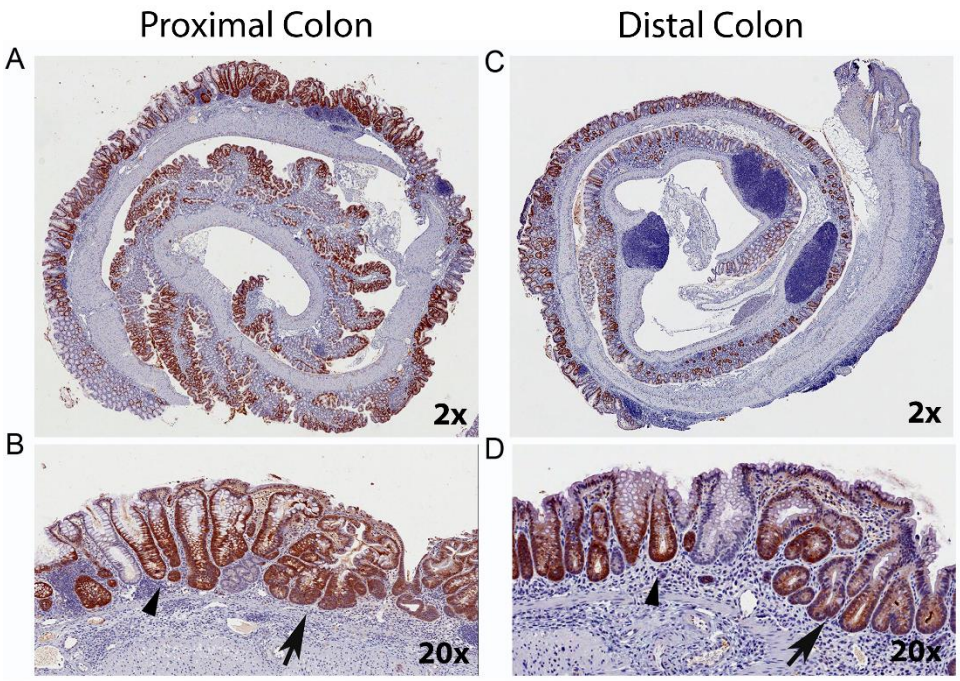

Figure 2.3 Immunohistochemical labeling for βgal in proximal and distal colon of DSS-treated CAC$^{Tg/WT}$;Rosa26R$^{flox/WT}$;Apc$^{\Delta 580/WT}$ mice. Animals were treated with 2% DSS for 5 days, and the tissues were collected at 10 days after stopping DSS treatment. Representative images were generated using an Aperio ScanScope digital slide scanner and are presented for proximal colon (A, B) and distal colon (C, D). The top pictures are at 2X magnification and the bottom pictures are at 20X magnification. Arrow head = normal crypt structure adjacent to a healing area. Arrow = crypt fission phenotype within a healing area.

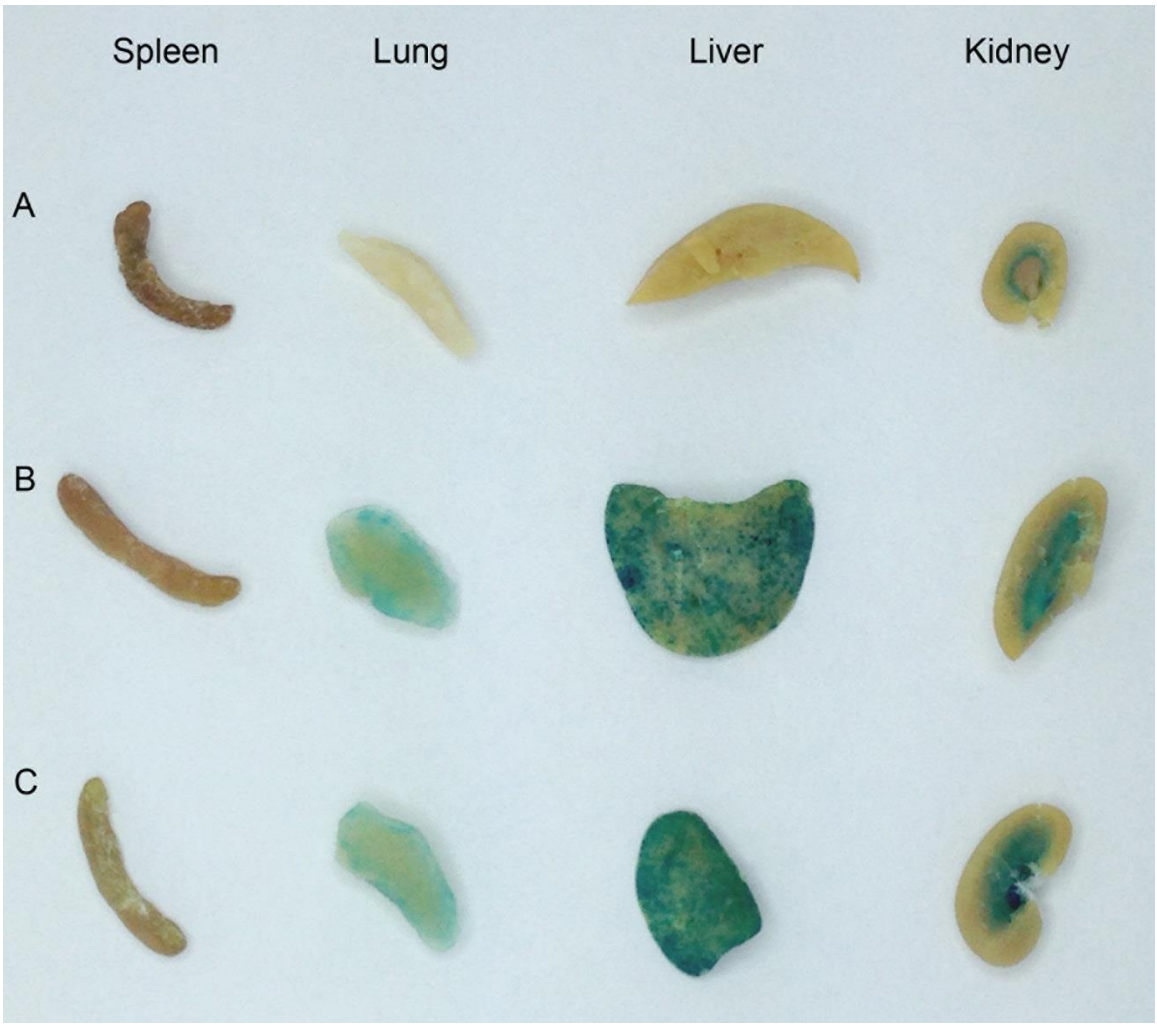

Figure 2.4 βgal expression in extra colonic tissues (spleen, lung, liver and kidney) was not induced by DSS treatment. Tissues were harvested, prepared, and βgal activity was detected as described for the colon. Blue staining represents βgal enzymatic activity. (A) Negative control: mice lacking the Cre-recombinase transgene. (B) βgal activity in $CAC^{Tg/WT}$;*Rosa26R* $^{flox/flox}$ transgenic mice without DSS treatment. (C) βgal activity in $CAC^{Tg/WT}$; *Rosa26R* $^{flox/flox}$ mice 10 days after completing a 5 day course of 1.35 % DSS.

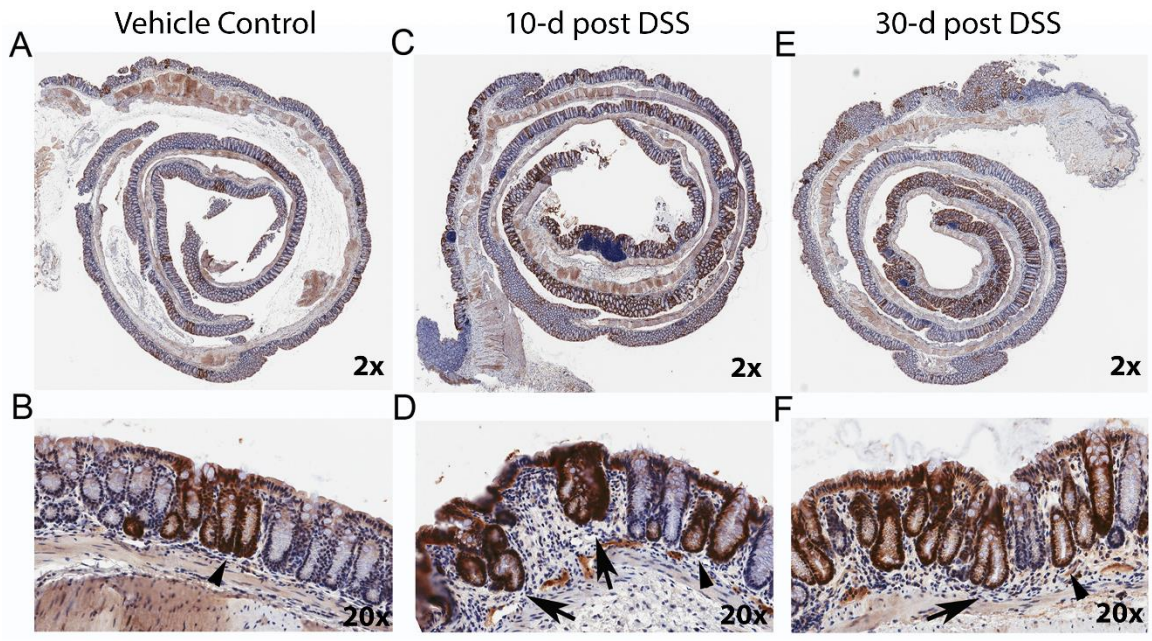

Figure 2.5 After DSS treatment transgene expression is increased in regenerating crypts and is sustained after healing of ulcers in distal colon. (A, B) Representative images of βgal expression level in the distal colon of a $CAC^{Tg/WT}$;$Rosa26R^{flox/flox}$ mouse treated with vehicle under 2X (A) and 20X (B) magnification. Crypt base labeling is highlighted with arrow head. (C, D) Images of βgal labeling in a $CAC^{Tg/WT}$;$Rosa26R^{flox/flox}$ mouse 10 days after the end of treatment with 1.35% DSS under 2X (C) and 20X (D) magnification. (E, F) Images of βgal labeling in a $CAC^{Tg/WT}$;$Rosa26R^{flox/flox}$ mouse 30 days after the end of treatment with 1.35% DSS under 2X (E) and 20X (F) magnification. In (C, D, E, F), Arrow = βgal positive crypts undergoing regeneration. Arrow head = β-gal positive crypts with normal phenotype adjacent to a healing area. All the images were generated using an Aperio ScanScope digital slide scanner.

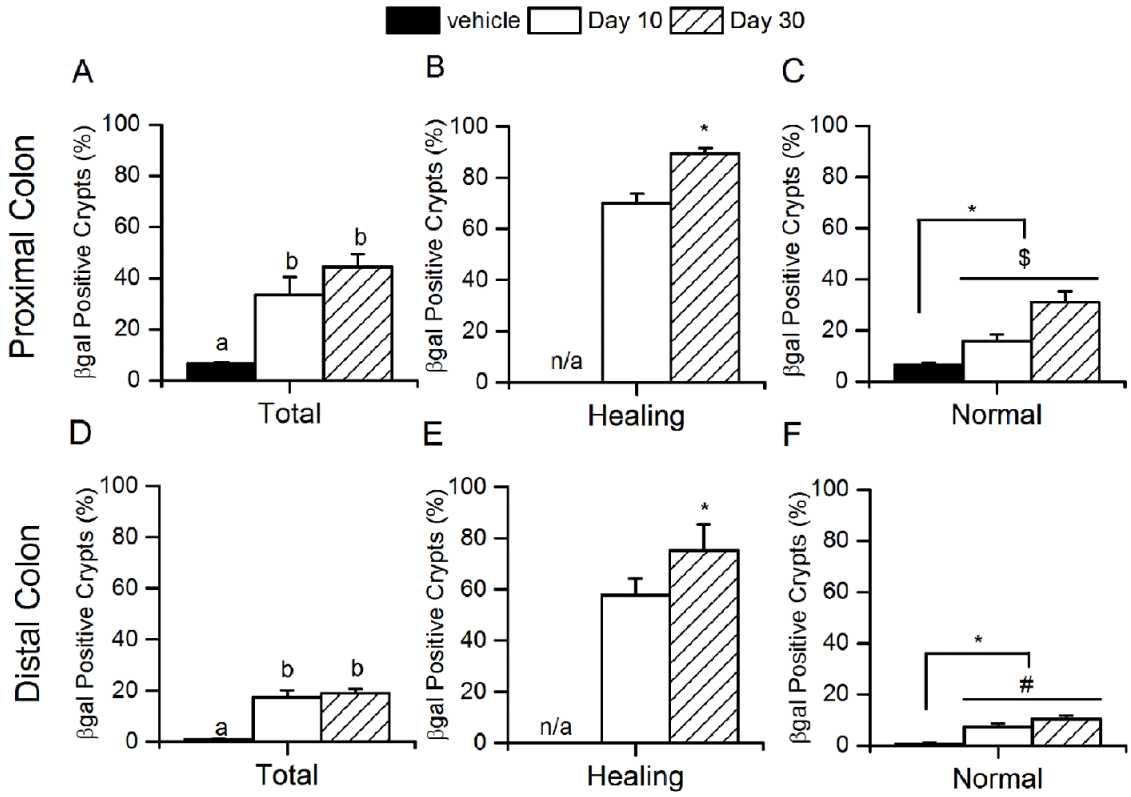

Figure 2.6 The percentage of β-gal positive crypts increases after DSS treatment and is sustained after regeneration in the colon of CAC$^{Tg/WT}$;*Rosa26R*$^{flox/flox}$ mice. (A, D) The percentage of β-gal positive crypts was measured in proximal (A) and distal (D) colon at 10 or 30 days after ending DSS treatment. Means with different letters are significantly different (Bonferroni, $p < 0.05$). (B, E) The percentage of β-gal positive crypts in mucosa that exhibits a healing phenotype in the proximal (B) and distal (E) colon. Student t-tests were used to compare values between day 30 and day 10 (* $p<0.05$). (C, F) The percentage of βgal positive crypts in normal mucosa was measured in proximal (C) and distal (F) colon. Planned orthogonal comparisons were performed (* $p<0.05$, day 0 vs post-treatment values; $ $p<0.05$, day 10 vs day 30; # $p<0.10$, day 10 vs day 30). Bars represent the mean ± SEM of 8 animals per group.

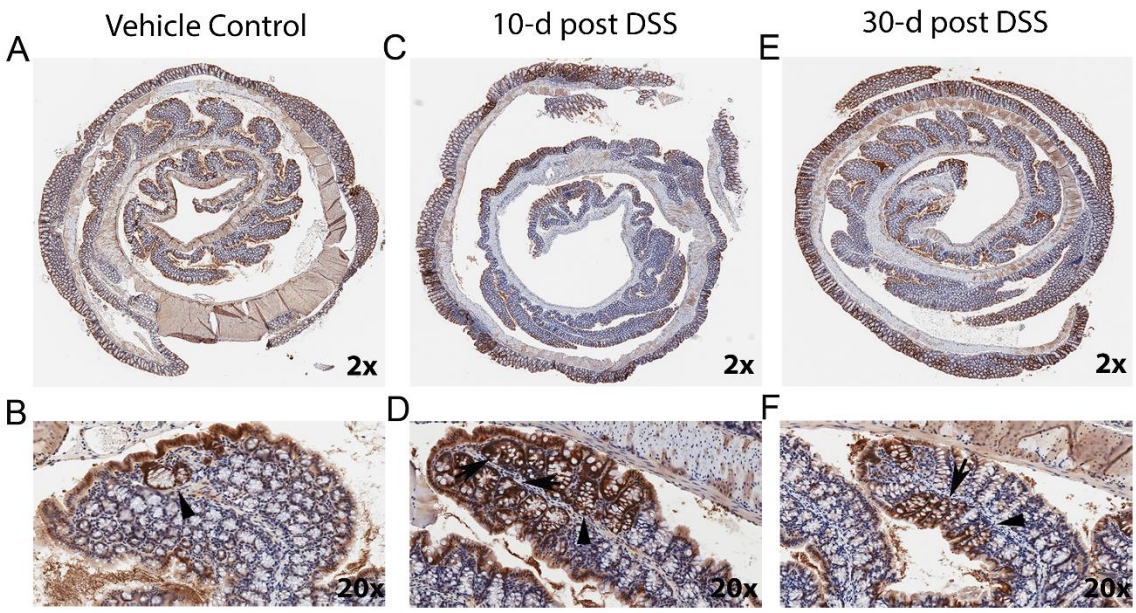

Figure 2.7 Transgene expression is increased in regenerating crypts and is sustained after healing in proximal colon. (A, B) Representative images of βgal expression level in the proximal colon of a $CAC^{Tg/WT}$;$Rosa26R^{flox/flox}$ mouse treated with vehicle under 2X (A) and 20X (B) magnification. Crypt base labeling is highlighted with arrow head. (C, D) Images of βgal labeling in a $CAC^{Tg/WT}$;$Rosa26R^{flox/flox}$ mouse 10 days after the end of treatment with 1.35% DSS under 2X (C) and 20X (D) magnification. (E, F) Images of βgal labeling in a $CAC^{Tg/WT}$;$Rosa26R^{flox/flox}$ mouse 30 days after the end of treatment with 1.35% DSS under 2X (E) and 20X (F) magnification. In (C, D, E, F), Arrow = βgal positive crypts undergoing regeneration. Arrow head = β-gal positive crypts with normal phenotype adjacent to a healing area. All the images were generated using an Aperio ScanScope digital slide scanner.

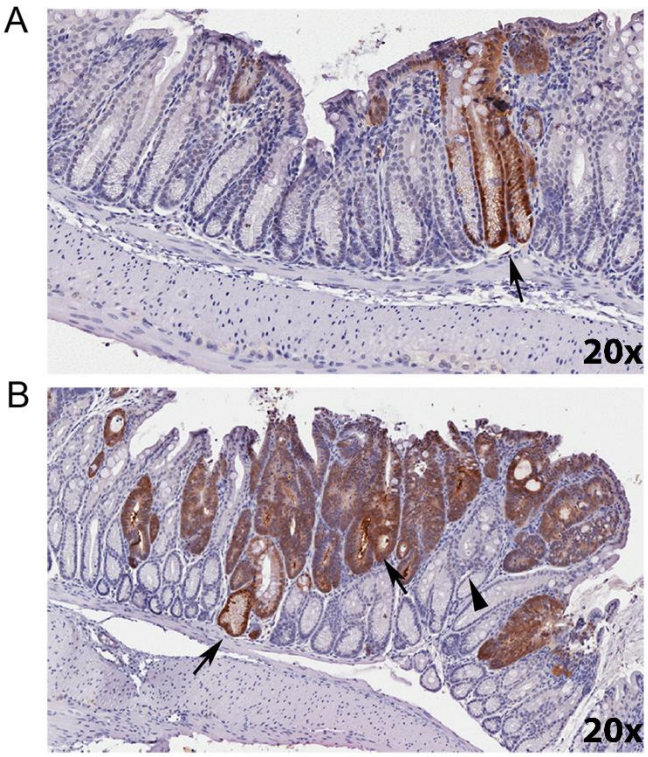

Figure 2.8 Colon tissue with an adenomatous phenotype show high βgal expression level in 4-week-old $CAC^{Tg/WT}$; $Rosa26R^{flox/WT}$; $Apc^{\Delta 580/\Delta 580}$ mice. (A) βgal expression in a normal area of the distal colon. Arrow = βgal labeling in a normal crypt. (B) βgal expression in distal colon adenomatous phenotype. Arrow = adenomatous phenotype with βgal positive labeling. Arrow head = adenomatous phenotype with βgal negative labeling. Images were generated using an Aperio ScanScope digital slide scanner. Magnification: 20X.

CHAPTER 3. VITAMIN D RECEPTOR-DEPENDENT SIGNALING IN BOTH COLONIC EPITHELIAL AND NON-EPITHELIAL CELLS PROTECTS MICE FROM DEXTRAN SULPHATE SODIUM-INDUCED COLITIS

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3.1 Abstract

Low vitamin D status is associated with an increased risk of inflammatory bowel disease in humans while low dietary vitamin D intake increases the severity of dextran sulphate sodium (DSS)-induced colitis in mice (Pilot study). However, it is not clear which cells in the inflamed colon are vitamin D targets and contribute to the enhanced colitis seen in vitamin D deficiency. We tested the hypothesis that the vitamin D receptor (VDR) has independent roles in colonic epithelial cells and in non-epithelial cells (primarily infiltrating immune cells) during experimental colitis. We further conducted two studies where VDR was deleted from either colon epithelial cells (study 1) or non-epithelial cells (Study 2) in mice with experimental colitis. 8-wk-old mice were treated with 1.35% DSS for 5 days then killed 10 day after completing the DSS treatment. Body weight (BW) loss, disease activity index (DAI) and spleen weight were assessed as systemic measures of colitis severity. Colitis was measured histologically in colon tissue and scores were determined for epithelial damage and inflammation. Colon tissue cytokine gene expression levels were analyzed by RT-PCR. In study 1, DSS-induced changes in BW, DAI and spleen weight were not increased by colon epithelial cell-specific VDR deletion (CAC; VDR KO). However, colon damage and inflammation at 10 day post DSS were still elevated in CAC; VDR KO mice compared to controls although DSS induced expression of colonic NOS2, TNF-α and IL-1β mRNA were not enhanced in those mice. The data indicates that VDR deletion in colon epithelial cells protect against DSS-induced local tissue damage but does not affect the systemic response. In study 2, DSS treated mice lacking VDR in non-epithelial cells (HV2; VDR KO) had more severe BW loss, DAI and spleen enlargement than controls. Histologic

analysis showed that the HV2; VDR KO mice also had more severe colon damage and inflammation than controls at day 10. DSS treatment induced colonic expression of Mϕ-associated pro-inflammatory mediators (i.e. TNF-α, NOS2 and IL-1β) and this response was significantly higher in the colon of HV2; VDR KO mice. These data suggest that VDR deletion in Mϕs contributes to the robust pro-inflammatory response seen both in the colon and systemically. The major finding of this work is that vitamin D-mediated protection from experimental colitis is dependent upon VDR signaling in both colon epithelial cells and in Mϕs, and the Mϕs are the primary vitamin D target cells during colitis.

3.2 Introduction

Inflammatory bowel disease (IBD) refers to a chronic and relapsing inflammatory response in the gastrointestinal tract, and it can be further categorized as Crohn's Disease (CD) or Ulcerative Colitis (UC) in humans (*1*). Even though the etiology of IBD is complex and unclear, IBD disease risk is associated with multiple risk factors, including genetics, life-style, infection, and immune system disturbance (*2*). All of these factors, individually or combined, may affect the disease initiation, development, recovery and relapse. Intestinal epithelial cells and immune cells play crucial but distinct roles in maintaining intestine tissue homeostasis and preventing IBD development (*3*). Epithelial cells form an intestinal barrier that prevents the infiltration of luminal bacteria and foreign antigens into the body. Impaired barrier function (e.g. loosened tight junctions or increased epithelial cell death) triggers a local immune response and induces chronic inflammation in the gut (*4, 5*). Immune cells are needed to maintain immune tolerance,

defend against infection and facilitate immune resolution and mucosa healing (*6*). Disturbance of the biological functions in either cell compartment increases the risk of IBD.

Vitamin D is a fat-soluble pre-hormone that can be absorbed from diet or be synthesized in skin from 7-dehydrocholesterol under UV exposure (*7*). The biological function of vitamin D is dependent on its active form, 1,25 dihydroxyvitamin D ($1,25(OH)_2D$), which binds to the vitamin D receptor (VDR) and regulates gene transcription (*8*). $1,25(OH)_2D$ has various biological functions, such as inducing calcium absorption, modulating immune response and preventing cancer development (*9*). Growing epidemiological evidence has shown that low vitamin D status is associated with increased IBD risk, and that vitamin D supplementation significantly improved human IBD outcomes (*10-15*). Similarly, in experimental colitis models low vitamin D intake or VDR deletion caused more severe colon damage, inflammation and systemic colitis disease activity in mice (*16, 17*). Moreover, human VDR polymorphism (i.e. ApaI, BsmI, FokI and TaqI) is associated with both UC and CD risk (*18-21*). These results indicate that vitamin D deficiency as a causal lifestyle factor in IBD development.

VDR is expressed in both intestinal epithelial cells and immune cells, and thus the inhibitory effects of vitamin D on IBD may be through either or both of these cell compartments. Previous studies showed that vitamin D signaling is crucial for maintaining epithelial cell tight junction protein expression and localization, preventing cytokine induced epithelial cell apoptosis, and inducing autophagy in Paneth cells; all of which are related to intestinal epithelial cell mediated protection from colitis (*22-24*). However, vitamin D also has anti-microbial and immune suppressive effects that in a

diverse group of immune cells (*25*). Therefore in our study, we used two novel animal models to test the independent role of VDR-dependent signaling in the two cell compartments on DSS-induced colitis. Our data demonstrated that VDR in both colon epithelial cells and non-epithelial cells (primarily immune cells) protects against DSS-induced colon tissue damage, but only VDR deletion in immune cells caused delayed healing and more robust systemic colitis response.

3.3 Materials and Methods

3.3.1 Animals

CAC/DSS mice were generated and characterized by our laboratory (*26, 27*). This mouse model has Cre-recombinase (Cre) transgene expression limited in colon, inducible Cre transgene mediated recombination during DSS-colitis and sustained gene inactivation during healing (*26, 27*). The novelty of the CAC/DSS mouse model made it suitable for studying the role of a gene in colon epithelial cells during healing. *Rosa26R*, $Apc^{CKO/CKO}$ and $Vdr^{flox/flox}$ were used to generate breeders in our experiments and they were all previously described (*26-28*). HA-hVDR–Expressing Transgenic Mice ($HV2^{Tg/-}$) express an HA-tagged human VDR (hVDR) exclusively in the intestinal epithelium (*29*). Global VDR knockout mice (VDR KO) were described elsewhere (*30*). All breeding colonies were maintained on a C57Bl/6J background. They were housed individually, given a standard chow diet and water ad libitum, and exposed to a 12-h light/12-h dark cycle in the Life Science Animal Facility at Purdue. All mouse experiments were approved by the Purdue Animal Care and Use Committee.

In the pilot study and study 1, CAC$^{Tg/-}$;*Rosa26R*$^{flox/-}$;*Apc*$^{\Delta 580/WT}$ (CAC) mice were generated by breeding CAC$^{Tg/-}$;*Rosa26R*$^{flo/flox}$ and *Apc*$^{CKO/CKO}$ mice (*27*). Experimental mice in study 1 with colon epithelial cell VDR inactivation, CAC$^{Tg/-}$;*Rosa26R*$^{flox/-}$;*Apc*$^{\Delta 580/WT}$;*Vdr*$^{\Delta ex2/\Delta ex2}$ (CAC; VDR KO) mice, were generated by breeding CAC$^{Tg/-}$;*Rosa26R*$^{flox/flox}$;*Vdr*$^{\Delta ex2/\Delta ex2}$ and *Apc*$^{CKO/CKO}$;*Vdr*$^{flox/flox}$ mice. CAC$^{Tg/-}$;*Rosa26R*$^{flox/flox}$;*Vdr*$^{\Delta ex2/\Delta ex2}$ mice were generated by first establishing a breeding colony of *Rosa26R*$^{flox/flox}$;*Vdr*$^{flox/flox}$ double transgenic mice, then sequentially backcrossed to CAC$^{Tg/-}$;*Rosa26R*$^{flox/flox}$ mice. *Apc*$^{CKO/CKO}$;*Vdr*$^{flox/flox}$ mice were generated by sequential backcrossing *Apc*$^{CKO/CKO}$ to *Vdr*$^{flox/flox}$ mice . In study 2, HV2$^{Tg/-}$ and VDR KO mice were bred to generate experimental mice HV2$^{Tg/-}$; VDR$^{-/-}$ (HV2; VDR KO). These mice had VDR recovered in intestine but deleted from all other tissues including the entire immune system. Control mice HV2$^{Tg/-}$ (HV2) were maintained by crossing HV2$^{Tg/-}$ with C57BL6/J wild type mice. Genotypes were determined by analyzing tail genomic DNA by standard PCR methods as previously described for CAC (*26*),*Rosa26R* (*31*), floxed *Apc* allele (*26*), floxed VDR allele (*28*), HV2 (*29*) and VDR KO (*32*).

3.3.2 Study Design

Pilot study: Experimental mice (CAC) were weaned at 3 weeks of age and fed the AIN93G diets containing either 1000 IU or 0 IU vitamin D_3/kg supplement and deionized water *as libitum* (n=112 per diet) (*33*). Mice in each diet group were randomly assigned into one of four dextran sulfate sodium (DSS) treatment groups (0%, 0.65%, 1.35% and 2%, n=28 per dose balanced by gender). DSS (MW = 36,000-50,000 Da, MP Biomedicals, LLC, Solon, OH)**)** was diluted in deionized water (w/v) to the appropriate concentrations**.** At 8 weeks of age, mice received the DSS solution in place of water *ad*

libitum for 5 consecutive days. Afterwards, DSS was replaced with deionized water and this was provided until the day of sacrifice. Half of the mice in each group were sacrificed 2 days after ending the DSS treatment and half were sacrificed 10 days after ending DSS treatment. All mice were fasted overnight before harvest. Body weight (BW), rectal bleeding and feces consistency of the mice were recorded daily from the start of DSS treatment until the day of harvest. Spleen weight of each mouse was recorded at the end of the study.

Study 1: 64 CAC (32 female and 32 male) and 64 CAC; VDR KO (32 female and 32 male) mice were weaned at 21 days old. All the experimental mice were fed standard AIN93G diet containing 1000 IU vitamin D_3/kg (*33*). Mice were treated with either vehicle (0% DSS) or 1.35% DSS dose (n=64 per dose balanced by genotype and gender) at 8 weeks old for 5 day, followed by deionized water until the harvest. Same as the pilot study, mice were harvested at 2 day or 10 day after ending DSS treatment, and the BW, rectal bleeding, feces consistency and spleen weight were measured in the same way. At each harvest time point, tissue from half of the mice (n=8, balanced by gender, genotype and DSS dose) were collected and preserved by formalin fixation, processed routinely, and embedded in paraffin. Colonic tissues from these paraffin blocks were used for histopathologic examination. Tissue from the other half of the animals (n=8) were harvest for gene expression endpoints.

Study 2: 64 HV2 (32 female and 32 male) and 64 HV2; VDR KO (32 female and 32 male) mice were used in this study. The study design was the same as described in study 1.

3.3.3 Live phase observation: daily BW loss and disease activity index

Mice were weighed daily from the start of DSS treatment (day -5) until the harvest day. Mice rectal bleeding and feces consistency were also evaluated daily. Each mouse was assigned a daily disease activity index (DAI) score as previously described by Cooper *et. al*. (*34*). BW loss score is a 0-4 scale score describing the percentage of BW loss daily (score 0= <1% loss from baseline; score 1= 1%-4.9% loss from baseline; score 2=5%-9.9% loss from baseline; score 3=10%-20% loss from baseline; score 4= >20% loss from baseline). Rectal bleeding score is described by three values scaled from 0 to 4 (score 0= normal color feces; score 2= Feces are red to dark red-brown, but there is no significant perianal blood staining or blood spots in the cage bedding; score 4= Perianal blood staining and/or blood spots in the cage bedding). Feces consistency score is also described by three values scaled from 0 to 4 (score 0= Normal formed feces; score 2= Feces loosely formed a soft that do not stick to anus; score 4= Feces fail to form any type of fecal pellet and/or there is perianal fecal staining). Daily DAI of each mouse was calculated as the average value of the three individual scores.

3.3.4 Necropsy

Mice were fasted for approximately 12 hours before harvest. Mice were weighed and then anesthetized with ketamine/xylazine (2 mg/g and 0.2 mg/g respectively). The blood was collected by cardiac venipuncture. Spleen and large intestine were carefully dissected and removed. The spleen was weighed and the colon length was measured. The colon was cut open along its mesenteric border from the anus to the distal end of cecum. The mucosa was carefully rinsed with cold phosphate buffered saline (PBS).

3.3.5 Serum Metabolite Assays

Serum concentrations of 25(OH)D (n=16-19/group) and 1,25$(OH)_2$D (n= 4-8/ group) were determined as per the manufacturer's instructions using radio immunoassays (Immunodiagnostic Systems, Scottsdale, AZ). Total serum calcium (n=8-10/diet and DSS group) was determined using a QuantiChrom Calcium Assay Kit (BioAssay Systems, Hayward, CA) per the manufacturer's instructions.

3.3.6 Formalin-Fixed Paraffin Embedded Tissue Collection

The colon was transected at its midpoint to separate the proximal and distal colon. Each section was split longitudinally and Swiss-rolled (*35*), resulting in two rolls of proximal colon and two rolls of distal colon per mouse. The spleen was cut along its longest axis before fixation. Tissues were immersion fixed for 48 hours in 10% neutral buffered formalin at 4°C before being transferred to 70% ethanol. Tissues were routinely processed and embedded in paraffin within 7 days of collection at the Purdue Histology and Phenotyping Laboratory.

3.3.7 Histology and Image Analysis

Colonic Swiss roll sections were cut at 4 µm thick and stained with hematoxylin and eosin. The sections were blindly evaluated by FW under the supervision of a board-certified veterinary pathologist (PWS). Quantitative morphology of colon was assessed using ImageScope software (Aperio, Vista, CA). Scores were assigned to the distal colon for extent of mucosal damage and immune cell infiltration. The criteria used for these histologic component scores are summarized in Table 3.1.

Spleen samples were cut and stained using the same method as colon. Spleen H&E stained images were taken from white light microscope (Leika, Germany) under an 8x

eyepiece and a 4x objective. About 60% of total area was captured from each spleen H&E stained sample. Spleen total area and white pulp area per image were measured directly from Image J, while red pulp area was calculated by subtracting the white pulp area from the total area. Percentage red pulp was calculated by dividing red pulp area by the total area.

3.3.8 Gene expression

Colonic RNA was isolated from either whole colon tissue (Study 1) or a colonic mucosa scraping (Study 2) using the Qiagen RNeasy kit. Spleen RNA was isolated using Direct-zol RNA kits from Zymo research (catalogue No.R2050, Irvine, CA, USA). Purified RNA was reverse transcribed to make cDNA and analyzed for TNF-α, NOS2 and IL-1β gene expression. The primer sets we used were pre-made by Integrated DNA Technology (TNF-α Assay ID: Mm.PT.58.12575861; NOS2 Assay ID: Mm.PT.56a.43705194; IL-1β Assay ID: Mm.PT.58.41616450).

3.3.9 Statistical analysis

Data was analyzed using SAS enterprise 5.1 software (SAS Institute, Cary, NC). Histogram evaluation and Shapiro Wilk test of normality ($p>0.05$) were used to determine if data were normally distributed. Percent BW loss, serum metabolites and spleen weight were normally distributed and analyzed by 2-way ANOVA for the effects of genotype, DSS doses, and their interaction. DAI, histologic scores, and spleen NOS2 gene expression were analyzed by Kruskal-Wallis non-parametric ANOVA for a main effect of genotype. Colonic gene expression values were not normally distributed and required log10 transformation prior to analysis by two-way ANOVA. Multiple comparisons were done using Tukey adjustment. Spleen red pulp enlargement data was

analyzed by student's t-test. All data are reported as mean ± standard error of the mean (SEM) and the differences were considered significant at $p < 0.05$ and trend at $p<0.10$.

3.4 Results

3.4.1 Low vitamin D intake resulted in deficient vitamin D status and more severe colitis when treated with 0.65% or 1.35% DSS.

At the baseline, mice fed diet containing 1000 IU/kg vitamin D had an average serum 25(OH) D level of 175.45 ± 11.80 nmol/L (n=16) compared to the low vitamin D intake mice with an average serum 25(OH)D level of 16.86± 0.62 nmol/L (n=17). Interestingly, 1.35% and 2% DSS treatment in vitamin D sufficient mice significantly reduced the serum 25(OH)D level at 10 day after DSS termination (data not shown). The low vitamin D intake resulted in deficient vitamin D status in mice without causing hypocalcemia (serum calcium concentration =9.59 ± 0.29 mg/dL vs 10.87 ± 0.19 mg/dL in low vs high vitamin D mice, n=18/group). Moreover, the baseline body weight of mice with high or low vitamin D intake were not different (data not shown). DSS treatment induced body weight (BW) loss in mice in a dose-dependent manner, while the mice with low vitamin D intake had more robust response under 0.65% and 1.35% DSS treatment (Figure 3.1). Spleen weight also increased after DSS treatment in a dose-dependent manner. In the 1.35% DSS group, mice with deficient vitamin D status developed more robust spleen enlargement compared to the ones with sufficient vitamin D (Figure 3.2). Based on these results, the DSS dose in the following experiments was determined as 1.35%.

3.4.2 Colon epithelial cell VDR deletion did not increase BW loss or DAI in mice after DSS-induced colitis.

Mice with vehicle treatment had stable BW throughout the study, and no difference in BW change at baseline was observed between CAC and CAC; VDR KO mice (data not shown). DSS induced significant BW loss in both groups and the percentage of BW loss in the two groups was similar (Fig 3.3A). DAI was significantly increased by DSS in both CAC and CAC; VDR KO mice, reaching a peak value 2 days after stopping DSS (Fig 3.3B). However, CAC; VDR KO mice did not develop more severe DAI compared to CAC mice at either 2 or 10 d post-DSS (Fig 3.3B).

3.4.3 Non-epithelial cell VDR deletion increased DSS-induced BW loss and DAI.

Vehicle treated HV2 and HV2; VDR KO mice had stable and comparable BW during the experiment (data not shown), while DSS treatment caused significant BW loss in both groups (Fig 3.4A). HV2 mice had maximum BW loss of about 6% at 2 days after stopping DSS, and BW recovered at day 5 after stopping DSS (Fig 3.4A). In comparison, the HV2; VDR KO mice had more severe peak BW loss of greater than 10%, and their BW failed to recover until the end of the study (Fig 3.4A). Significantly more severe BW loss was observed in HV2; VDR KO mice from 4-8 days after DSS was removed compared to the control mice (Fig 3.4A). In addition, DAI of HV2; VDR KO mice was significantly higher than the controls from day -2 until the end of the study (Fig 3.4B).

3.4.4 DSS-induced spleen enlargement was increased in mice with VDR deletion in non-epithelial cells.

Spleen enlargement is often used as a systemic infection indicator and it is commonly seen in DSS-induced colitis (*17*, *36*). In study 1, both CAC and CAC; VDR KO mice had increased spleen weight as a percentage of BW at day 10 after stopping DSS, but no difference was seen between the two genotype groups (KO vs CAC = 0.49± 0.02% vs 0.52± 0.04%, n=16 vs 14 /group, p=0.71) (Fig 3.5A). In study 2, HV2; VDR KO had significantly more severe spleen enlargement induced by DSS compared to the HV2 controls (KO vs HV2 = 0.76± 0.04 % vs 0.52± 0.04 %, n=13/group, $p<0.0001$) (Fig 3.5B).

3.4.5 Mice with VDR deletion in both colon epithelial cells and non-epithelial cells had more severe DSS-induced colon damage.

In study 1, DSS treatment caused colon mucosa damage in both CAC and CAC; VDR KO groups, whereas CAC; VDR KO mice had more severe damage at both day 2 (p=0.0013 vs CAC) and day 10 (p=0.0283 vs CAC) after DSS termination (Fig 3.6A). Similar results were observed in study 2 where HV2; VDR KO mice had more robust colonic damage than HV2 mice at both time points (2 day, p=0.0794; 10 day, p=0.0155) (Fig 3.6B). This suggests that VDR in both colon epithelial cells and non-epithelial cells protects against DSS induced local tissue damage. Interestingly, CAC; VDR KO mice had significantly reduced colon damage scores at day 10 compared to day 2 after DSS was removed (p=0.0037) (Fig 3.6A), whereas colon mucosa damage of HV2; VDR KO

mice was steady from day 2 to day 10 post DSS (Fig 3.6B). This suggests that loss of VDR in non-epithelial cells delayed epithelial healing following DSS-induced colitis.

3.4.6 VDR deletion in both colon epithelial cells and non-epithelial cells caused more severe immune cell infiltration in the colon.

DSS-treatment also increased colonic immune cell infiltration in mice. In study 1, CAC; VDR KO mice had higher histological inflammation scores than controls at both day 2 ($p=0.0605$ vs CAC) and day 10 ($p=0.0036$ vs CAC) after stopping DSS (Fig 3.7A). Also, even though the colonic damage scores of CAC; VDR KO mice decreased with time, the inflammation scores were sustained from day 2 to day 10 (Fig 3.7A). Similarly, in study 2 HV2; VDR KO mice had significantly higher colon inflammation scores at day 10 than controls ($p=0.0142$), and did not decline from day 2 (2.13 ± 0.23) to day 10 (2.43 ± 0.30) (Fig 3.7B).

3.4.7 Mice lacking VDR in the Non-epithelial cell compartment had a more severe colonic Mϕ pro-inflammatory response.

Mϕ phenotypes are closely related to colon mucosa healing after DSS treatment, with M1 being detrimental and M2 being beneficial (*37, 38*). We hypothesized that the delayed colonic healing in HV2; VDR KO mice were caused by over-activated M1 Mϕ response. To test this hypothesis, we examined the colonic M1 Mϕ cytokine profile in our two studies. In study 1, NOS2 and IL-1β expression levels were slightly increased in the colon of DSS treated mice, and TNF-α expression level returned to normal by 10 days after DSS was removed. In contrast, the expression levels of the three genes in CAC;

VDR KO mice were not statistically different from those in CAC controls (Fig 3.8A-C). In study 2, TNF-α, NOS2 and IL-1β expression levels were significantly elevated by DSS treatment and this response was significantly higher in HV2; VDR KO mice compared to controls (Fig 3.8D-F). Since the three cytokines are predominantly produced by pro-inflammatory Mϕs, the data indicate that local inflammatory Mϕ over-activation is associated with increased colon mucosa damage and delayed healing seen in HV2; VDR KO mice.

3.4.8 The enhanced systemic response to DSS-induced colitis in HV2; VDR KO mice is due to over-activation of monocyte-Mϕ lineage in colitis.

All monocytes and a majority of Mϕs in the spleen are located in the red pulp, so we first tested whether VDR deletion in the non-epithelial compartment enlarged the spleen red pulp after DSS treatment. DSS treatment increased the red pulp by 20% in HV2 mice at 10 d post DSS compared, In contrast, the DSS-induced enlargement of red pulp in HV2; VDR KO mice was 45% and was significantly higher than that seen in HV2 control mice (Fig 3.9A). We further analyzed the spleen Mϕ pro-inflammatory response by measuring classically activated Mϕ marker NOS2 gene expression levels, and found that HV2; VDR KO mice had a significantly higher NOS2 expression in spleen compared to the controls (Fig 3.9B).

3.5 Discussion

Our pilot study independently confirmed that low dietary vitamin D intake caused more robust DSS-induced BW loss and spleen enlargement in mice. The goal of the

following two studies is to determine whether vitamin D signaling has an independent role in colon epithelial cells and non-epithelial cells during colitis. We conducted two experiments using mouse models with VDR deleted specifically from colon epithelial cells or non-epithelial cells (primarily infiltrating immune cells), and found that vitamin D signaling in both cell compartments protects against DSS-induced colon damage. However, only deleting VDR from non-epithelial cells caused delayed mucosa healing and more severe systemic colitis response. The increased colitis response was associated with Mϕ over-activation in colon and systemically. Therefore, we conclude that an important protective effect of vitamin D against colitis is through its regulation on Mϕ response, while its protective role on colon epithelial cells is secondary.

Low vitamin D status is often diagnosed in human IBD patients, and mice with low vitamin D intake or VDR deletion are more susceptible to developing experimental colitis (*16, 39-41*). Lagishetty et al. found that mice with vitamin D deficiency developed more severe BW loss and disease activity in DSS-colitis (*41*). This was consistent with a previous report that mice with deleted VDR had increased mortality rate, more severe BW loss and rectal bleeding in DSS-colitis (*16*). Moreover, these clinical symptoms of DSS-induced colitis were significantly alleviated by rectal or intragastric administration of $1,25(OH)_2D$ in mice (*16, 42*). In our pilot study, we were able to independently confirm that low vitamin D intake was the causal factor of more robust DSS-colitis in mice, reflected by more robust BW loss and spleen enlargement. By using four different doses of DSS, we found the most appropriate dose of 1.35% which was strong enough to cause colitis but not too strong to mask the vitamin D effect in our mouse model. Interestingly, Lagishetty et al. shown that 2.5% DSS reduced serum 25(OH)D levels in

mice with high vitamin D intake but did not reach statistically significant. By increasing the mouse number (n=8 vs 18/group), we observed a significant reduction of 25(OH)D serum level at 10 d after stopping 1.35% and 2% DSS treatment. In the following two studies, we observed the consistent results that non-epithelial cell VDR deletion in mice resulted in more severe BW loss and DAI as seen in vitamin D deficiency. However, these colitis symptoms were not observed in mice with VDR deleted from colon epithelial cells. This suggests that vitamin D-mediated protection against colitis is primarily through its regulation on non-epithelial cells, while blocking vitamin D signaling in colon epithelial cells is not sufficient to cause more severe clinical response in DSS-induced colitis.

Other studies showed that intestinal epithelial VDR protected mice from developing DSS induced systemic colitis response, whereas our data suggested the opposite (*24, 43*). This discrepancy can be explained by the different choices of animal models. For example, previous studies used mice expressing villin promoter modified Cre-recombinase, which deleted floxed VDR alleles from the entire intestine. Because VDR in the small intestine is crucial for active calcium absorption, these mice develope hypocalcemia when not fed a rescue diet (*24, 43*). Therefore, the robust colitis response may also be due to altered calcium metabolism (*24, 43*). To avoid this issue, Liu et al. then used an opposite model and investigated whether mice over-expressing intestinal VDR (Tg or Tg-KO) would be resistant to DSS-induced colitis relative to wild-type mice (WT) or VDR KO mice (KO) (*23*). They found that even through the mice expressing human VDR transgene in the intestine developed less severe DSS-colitis clinical score than WT, it only happened in the second round of DSS administration (each round=3%

DSS 7d+ water 7d) (*23*). More importantly, Tg-KO mice still had higher colitis clinical scores compared to Tg mice (*23*). This suggests that VDR in non-epithelial cells also has a role in the response to colitis. This is in agreement with our results. To improve the study design of previous studies, we used a DSS-inducible, large intestine specific transgenic mouse model to test the contribution of colon epithelial cell VDR to DSS-colitis prevention. This model ensured VDR inactivation in 40-50% colon crypts after 1.35% DSS administration, which was sufficient to inhibit VDR regulated vitamin D signaling in damaged colon epithelium (*23, 27*). In addition, transgene induced VDR inactivation was limited to the large intestine and thus would not interfere with body calcium homeostasis or VDR regulated biological functions in other tissues (*26, 27*). Based on our results, we conclude that colon epithelial cell VDR has impact on reducing DSS-induced colon damage locally.

Vitamin D plays an important role in maintaining colonic barrier function, potentially through maintaining epithelial cell permeability and autophagy, as well as inhibiting cytokine induced epithelial cell apoptosis (*22-24*). Our results are consistent with previous reports showing that deleting VDR from colon epithelial cells increased the severity of colon epithelial damage and immune cell infiltration at both 2d and 10d after ending 1.35% DSS treatment. Interestingly, a similar colonic response was also observed in our HV2; VDR KO mice. This suggests that VDR-mediated signaling in both colon epithelial cells and non-epithelial cells protects mice from developing colonic damage.

Even though deleting VDR from either colon epithelial cells or non-epithelial cells caused more severe colonic damage in mice, delayed mucosa healing was only observed in those with non-epithelial cell VDR deletion. Mucosa healing is a crucial IBD

therapeutic goal; delayed healing extends the disease duration and therefore increases the risk of IBD complications, such as colon cancer (*44*). Mucosa healing is a multi-step process, including colon epithelial cell restitution, proliferation and crypt regeneration. $1,25(OH)_2D$ (10 or 20 nM) was shown to induce Caco-2 cell migration in culture, and prevent inflammation induced human HCT116 cell apoptosis; both of which may facilitate mucosa healing after DSS-induced damage (*22, 23*). Previous animal studies focused on the role of VDR on disease severity/mucosa damage using animals that were sacrificed soon after experimental colitis was induced (*16, 17, 40*). To understand how VDR in colon epithelial cells promotes mucosa healing we used our unique CAC/DSS mouse model. In this model VDR deletion is limited to the site of epithelial damage (*26, 27*). By comparing the colonic damage score between 2 day and 10 day post DSS in CAC; VDR KO mice, we found that the mucosa healing was not affected when removing VDR from colon epithelial cells.

Mϕs, subtyped by M1 and M2, also affect the mucosa healing process (*37, 38*). Pro-inflammatory mediators (e.g. ROS and TNF-α) produced by M1 can induce colon epithelial cell death and thus prevent healing, whereas other immune factors (e.g. IL-10 and TGF-β) produced by M2 may facilitate mucosa healing by suppressing local pro-inflammatory response and inducing epithelial cell growth (*45-47*). Previous research in mouse models show that the M1/M2 Mϕ ratio increased during DSS administration and decreased during mucosa healing (*48, 49*). Similarly, in humans, IBD patients treated with infliximab or together with azathioprine (anti-TNF-α antibodies) had reduced total Mϕ numbers ($CD68^+$) and increased M2 Mϕ ($CD206^+$) numbers in mucosa, followed by improved mucosa healing (*50, 51*).

Mϕs have long been known as a vitamin D regulating target (*52, 53*). 1,25$(OH)_2$D significantly suppressed M1 Mϕ cytokine production in culture after LPS induction and in WT mice after DSS treatment (*42, 54-58*). Consistently, mice with global VDR deletion developed more robust colonic inflammation and increased M1 Mϕ cytokine levels (e.g. TNF-α, IL-1β and IL-12) when treated with DSS (*16*). Systemically, low vitamin D intake caused more severe spleen enlargement in DSS-colitis, and this was independently shown by Lagishetty et al. and us (*17*). Lagishetty et al. also demonstrated that this increased spleen size was not due to increased T and B cell numbers or over-activated adaptive immune cytokine response (*17*). Therefore, we hypothesized that the robust local and systemic response were mainly due to altered monocyte-Mϕ response. In agreement with these previous reports, our data showed that only deleting VDR from non-epithelial cells caused elevated colonic gene expression levels of M1 mediators (TNF-α, IL-1β and NOS2) during mucosa healing. Moreover, these mice also had significantly enlarged spleen red pulp (main location of monocytes and Mϕ) and M1 mediator NOS2 expression level in the spleen after DSS induced colitis. These data suggest that altered Mϕ response is the primary cause of more robust systemic colitis and delayed healing seen in vitamin D deficiency.

3.6 Conclusions

Our study has demonstrated that VDR-dependent signaling in both colon epithelial cells and immune cells protects against mucosa damage, but only VDR in immune cells protects mice from developing robust systemic response and delayed mucosa healing. We further demonstrated that monocyte/Mϕ lineage is a significant target of vitamin D action

during colitis. This suggests that the Mϕ is an important cell target for human IBD prevention and that treatment with vitamin D or vitamin D analogs could be developed reduce colitis. To further investigate the mechanism of how VD regulates monocyte/Mϕ response in colitis, investigators need to ask 1) whether vitamin D signaling can directly alter Mϕ phenotypes; 2) whether vitamin D regulates monocyte migration from bone marrow or recruitment from circulation into colon tissue; and 3) whether vitamin D regulates monocyte development in bone marrow during colitis. To confirm the role of Mϕ VDR in experimental colitis, a lineage specific VDR deletion model needs to be used to repeat the experiment discussed in this chapter. In the next chapter, I will mainly focus on the first follow-up question mentioned above: can vitamin D directly regulate Mϕ polarization and class switch in vitro?

3.7 References

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Table 3.1 Components of Histologic Grading Scheme for Colon Damage and Inflammation

Colon Epithelial Damage Score	
Score	**Description**
0	No mucosal damage
1	1-25% of mucosa ulcerated or healing
2	26-50% of mucosa ulcerated or healing
3	51%-75% of mucosa ulcerated or healing
4	Greater than 75% of mucosa ulcerated or healing
Colon Inflammation Score	
Score	**Description**
0	No Inflammation
1	Mild increase in lamina propria leukocytes
2	Moderate increase in lamia propria and submucosal leukocytes
3	Submucosal edema and moderate to marked increase in lamina propria and submucosal leukocytes
4	Extension of leukocytes into tunica muscularis and/or mild serositis
5	Extension of leukocytes through tunica muscularis with severe serositis

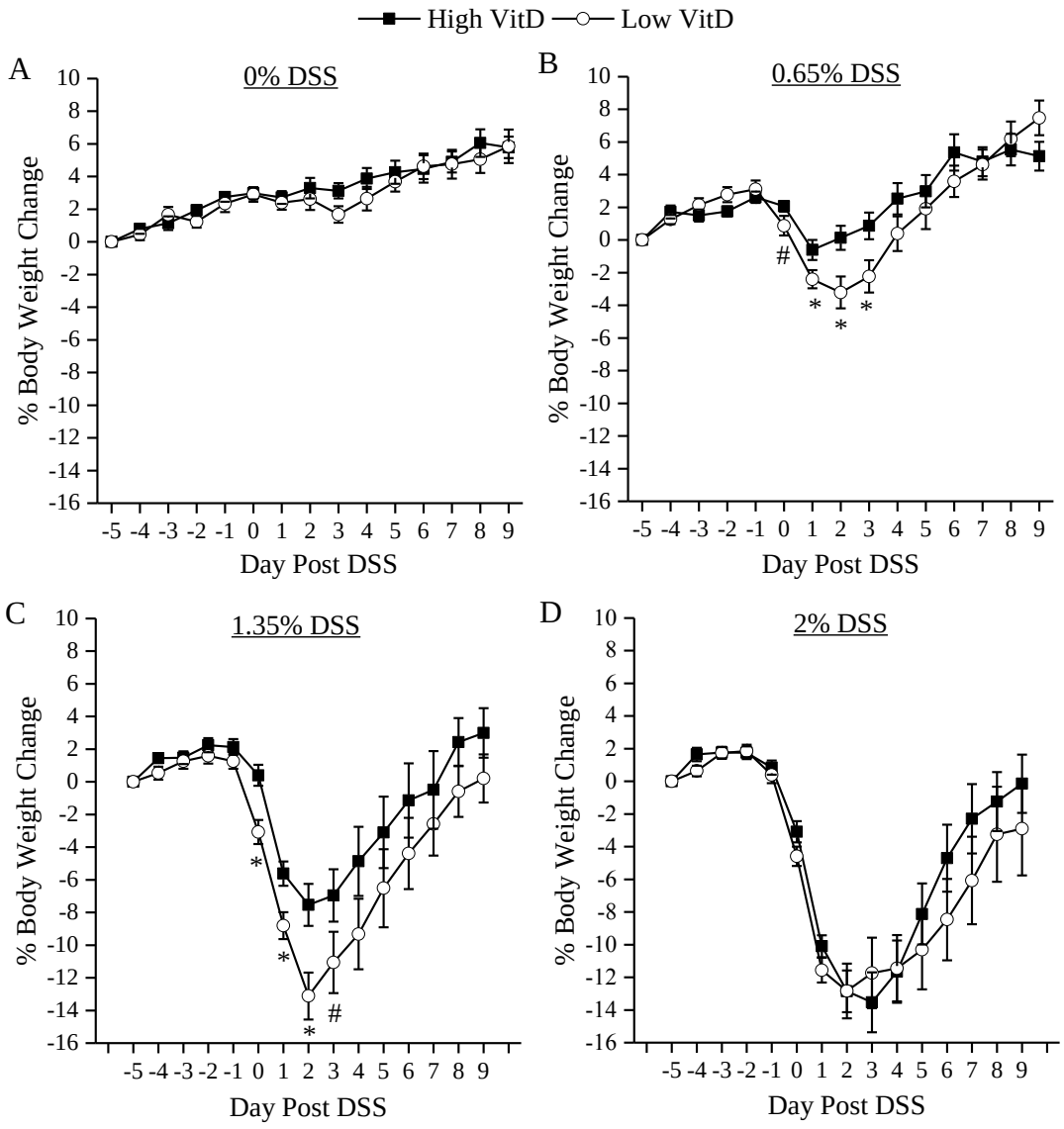

Figure 3.1 BW change of CAC mice fed high or low vitamin D diet during DSS-induced colitis and recovery. (A) Daily BW change of mice treated without DSS. (B) Daily BW change of mice treated with 0.65% DSS. (C) Daily BW change of mice treated with 1.35% DSS. (D) Daily BW change of mice treated with 2% DSS. (*, $p<0.05$; #, $p<0.10$).

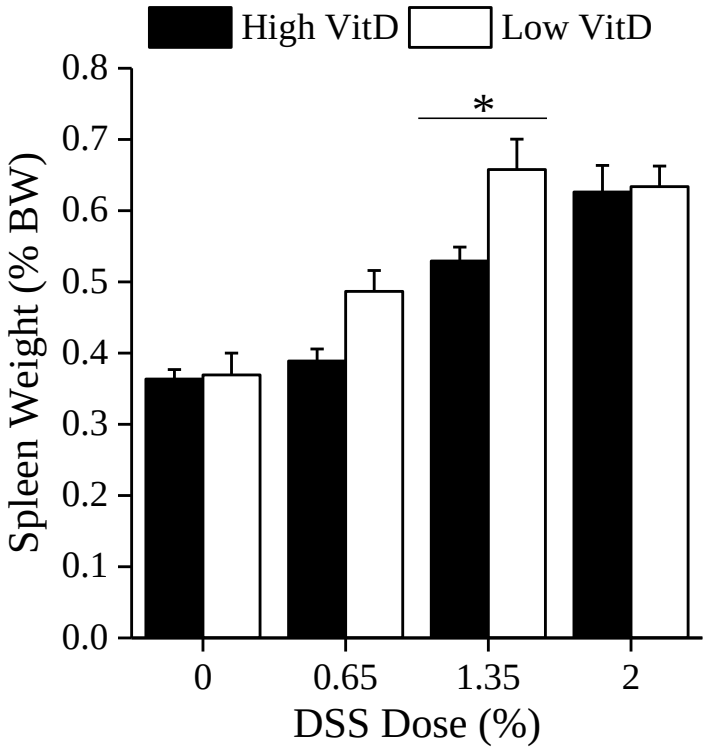

Figure 3.2 The average weight of the spleen as a percentage of BW in mice with high or low vitamin D intake 10 day after stopping DSS. (*, $p<0.05$).

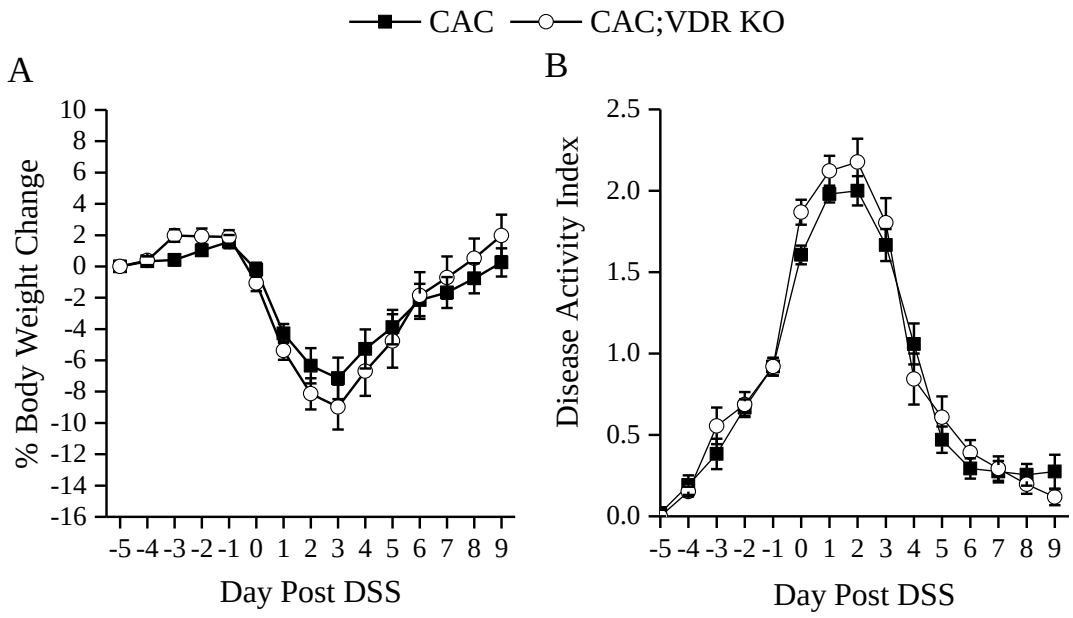

Figure 3.3 BW change and disease activity index of CAC and CAC; VDR KO mice during DSS induced colitis and recovery. (A) Daily BW change of mice treated with 1.35% DSS. (C) Daily disease activity index of mice treated with 1.35% DSS.

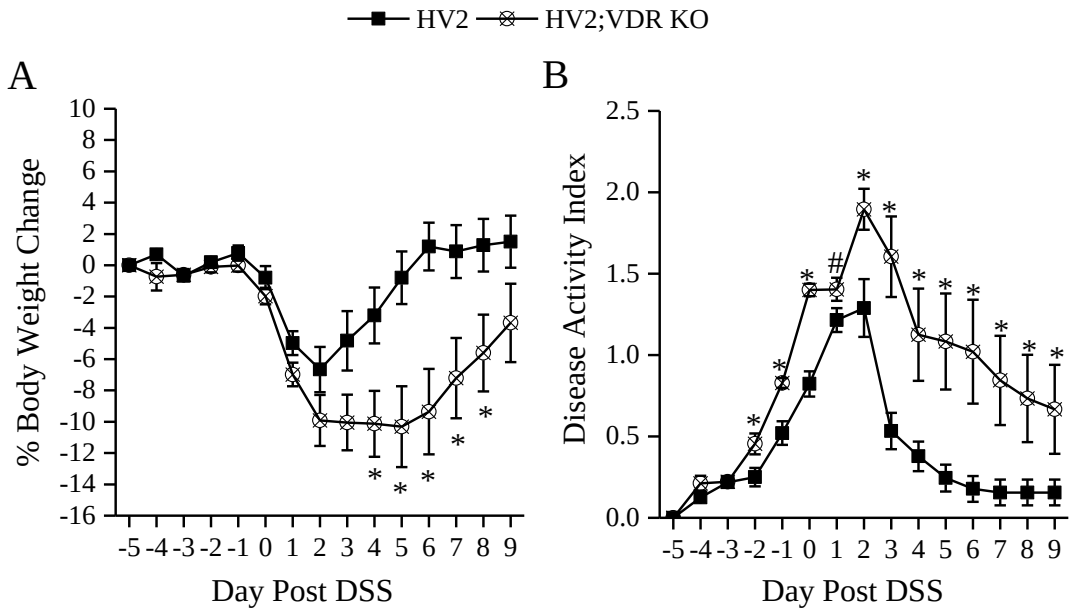

Figure 3.4 BW change and disease activity index of HV2 and HV2; VDR KO mice during DSS induced colitis and recovery. (A) Daily BW change of mice treated with 1.35% DSS. (C) Daily disease activity index of mice treated with 1.35% DSS (*, $p<0.05$, #, $p<0.10$ vs HV2).

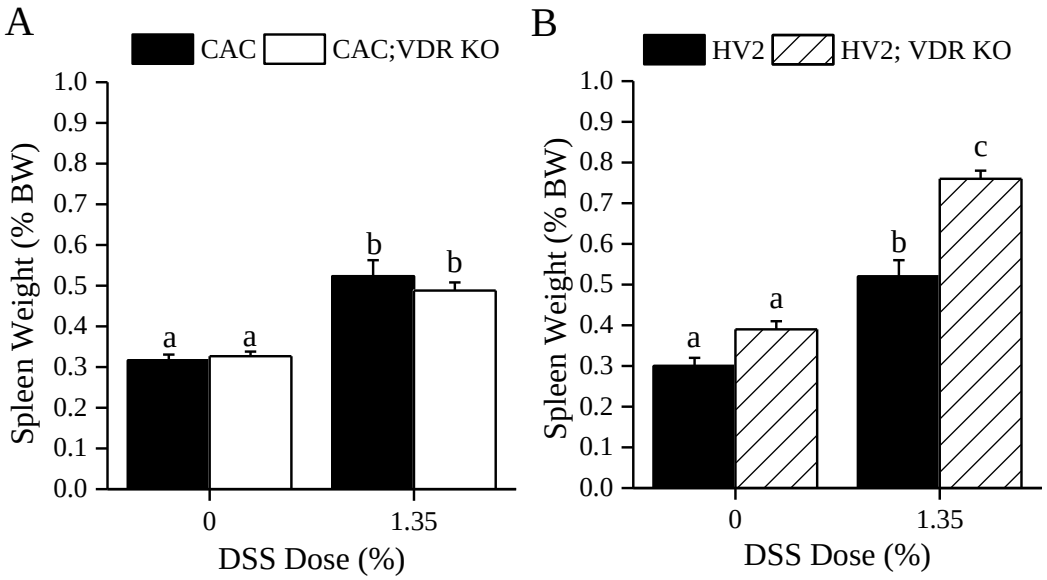

Figure 3.5 The average weight of the spleen as a percentage of BW in mice 10 day after stopping DSS. (A) CAC and CAC; VDR KO mice. (B) HV2 and HV2; VDR KO mice. (Different letters, $p<0.05$).

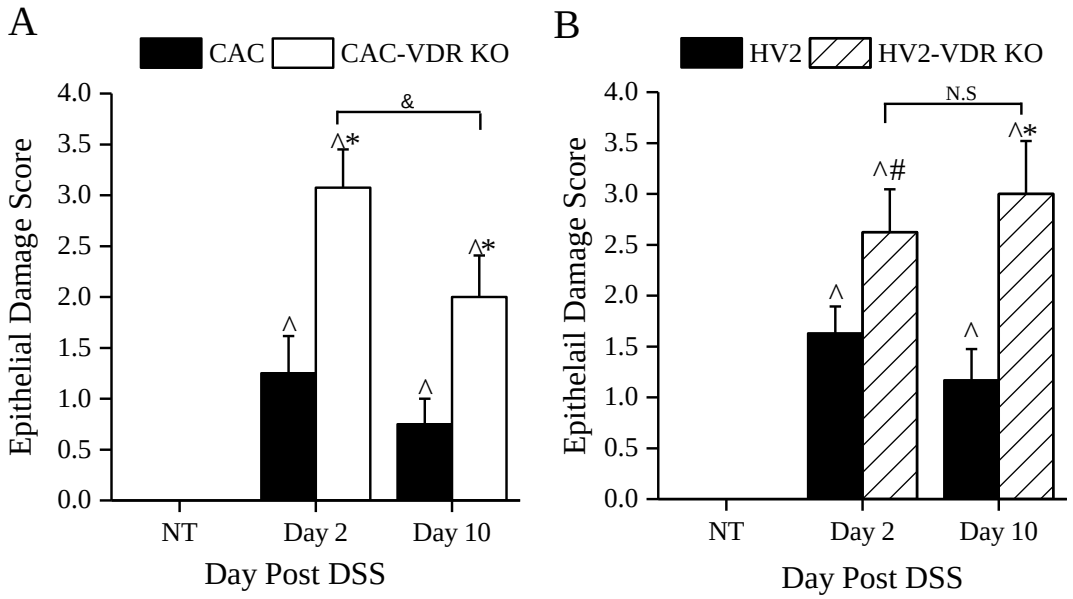

Figure 3.6 . Colon epithelial damage score in mice treated with vehicle (NT) and harvested at day 2 or day 10 post 1.35% DSS. Data represents histological score in distal colon. (A) Epithelial damage score in the colon of CAC and CAC; VDR KO mice (^, $p<0.05$ vs NT; *, $p<0.05$ vs CAC; &, $p<0.05$ vs Day 2). (B) Epithelial damage score in the colon of HV2 and HV2; VDR KO mice. (^, $p<0.05$ vs NT; *, $p<0.05$, #, $p<0.10$ vs HV2; N.S; not significant).

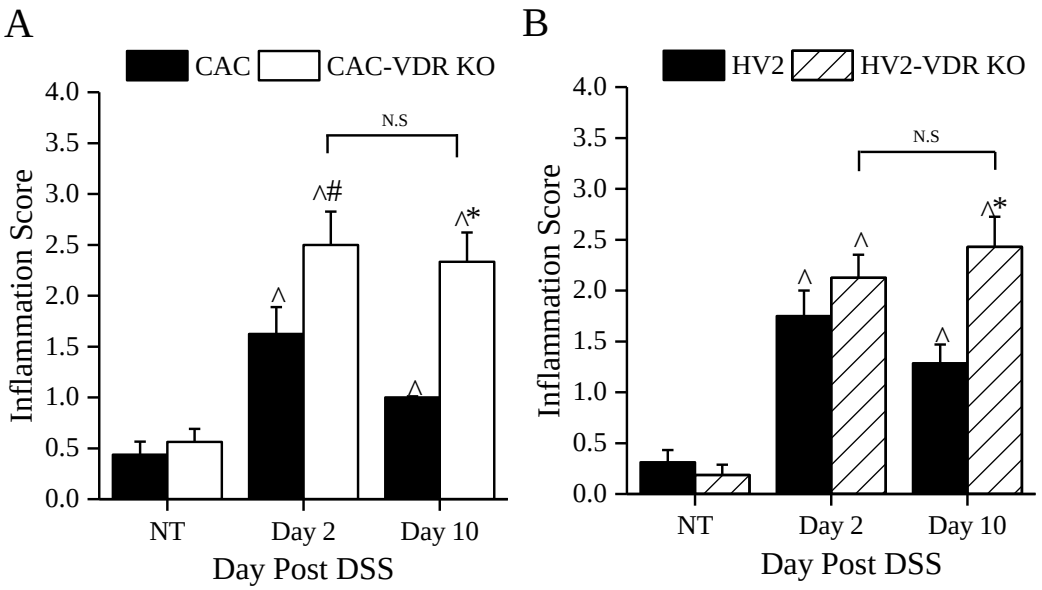

Figure 3.7 Colon inflammation score in mice treated with vehicle (NT) and harvested 2 day or 10 day post 1.35% DSS. Data represents histological score in distal colon. (A) Inflammation score in the colon of CAC and CAC; VDR KO mice (^, $p<0.05$ vs NT; *, $p<0.05$, #, $p<0.10$ vs CAC; N.S; not significant). (B) Inflammation score in the colon of HV2 and HV2; VDR KO mice. (^, $p<0.05$ vs NT; *, $p<0.05$ vs HV2; N.S; not significant).

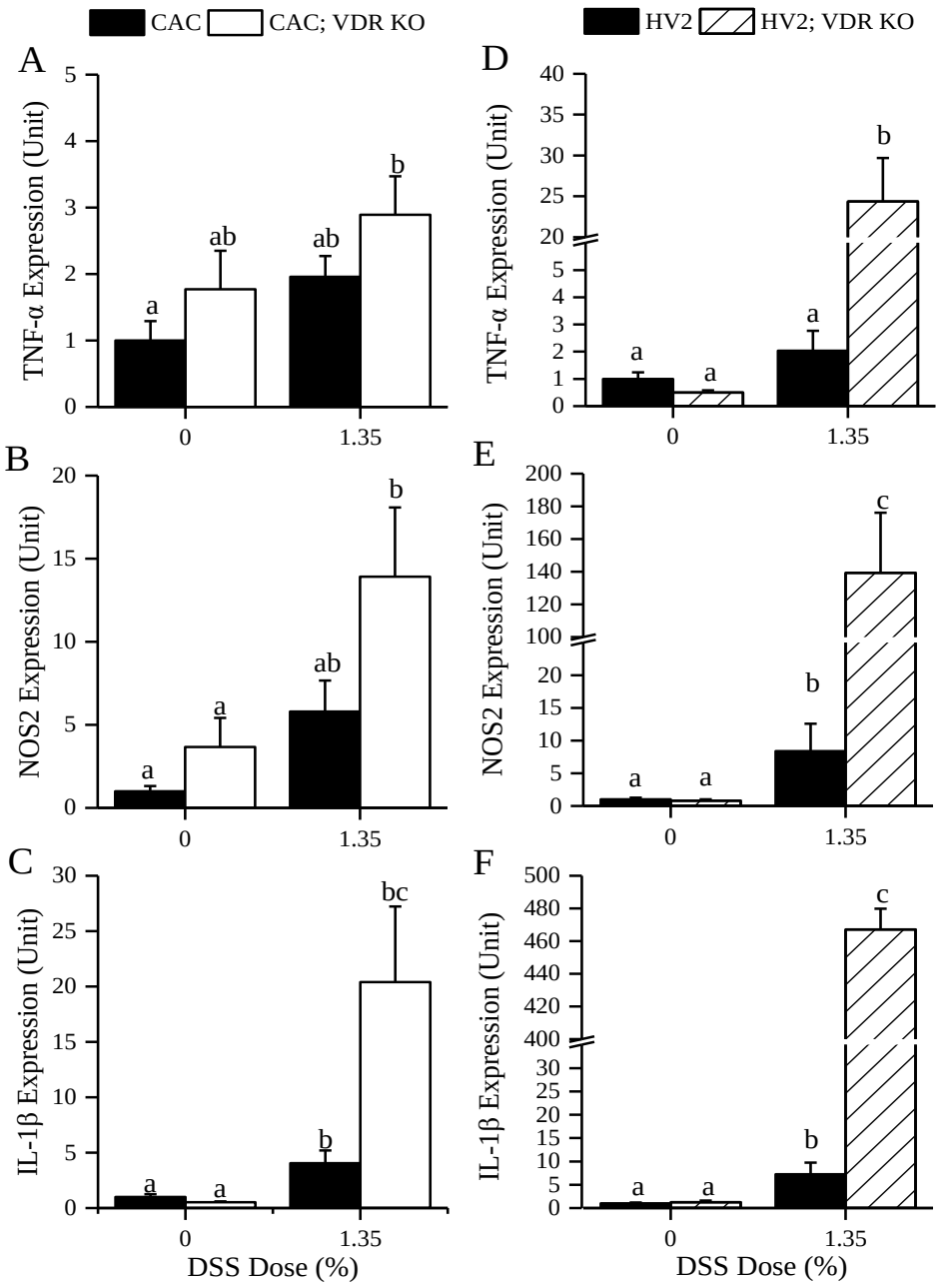

Figure 3.8 Mϕ activation related gene expression levels in the colon of mice 10 day after DSS was removed. Data represents gene expression in distal colon. TNF-α (A), NOS2 (B) and IL-1β (C) expression levels in the colon of CAC and CAC; VDR KO mice. TNF-α (D), NOS2 (E) and IL-1β (F) expression levels in the colon of HV2 and HV2; VDR KO mice. (Different letters, $p<0.05$).

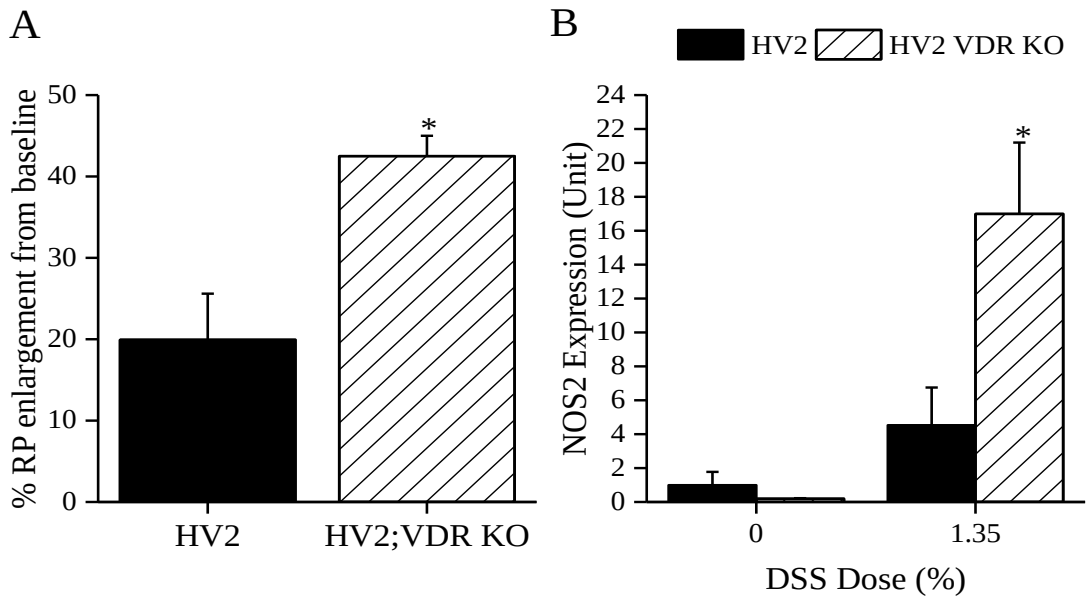

Figure 3.9 Spleen red pulp (RP) enlargement and Mϕ response at day 10 post DSS. (A) Percentage spleen red pulp (RP) enlargement at day 10 post 1.35% DSS from the baseline in HV2 and HV2; VDR KO mice. (B) NOS2 gene expression levels in spleen of HV2 and HV2; VDR KO mice 10 day after DSS was removed. (*, $p<0.05$ vs HV2).

CHAPTER 4. THE EFFECT OF 1A, 25 DIHYDROXYVITAMIN D ON MURINE PERITONEAL MACROPHAGE POLARIZATION AND PHENOTYPE SWITCH IN VITRO

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4.1 Abstract

Macrophage (Mϕ) activation and phenotypes are closely related to the progression of human inflammatory bowel disease (IBD). During colitis, classically activated Mϕs (M1) produce pro-inflammatory mediators and induce colon tissue injury, while alternatively activated Mϕs (M2) have anti-inflammatory features and facilitate colon mucosa regeneration. Vitamin D is an immune modulator which may suppress colitis by regulating Mϕ function, but the mechanism for this suppression is unknown. We tested the hypothesis that 1,25 dihydroxyvitamin D ($1,25(OH)_2D$) can directly induce M2 and suppress M1 Mϕ activation and phenotype using murine peritoneal Mϕ culture. We found that $1,25(OH)_2D$ treatment alone or combined with IL-4 significantly induced peritoneal Mϕs M2 marker Arg1 expression. $1,25(OH)_2D$ also suppressed IFN-γ and LPS induced M1 marker NOS2 expression by 20%. This suggests that $1,25(OH)_2D$ can induce polarization of Mϕs to the M2 subtype while inhibiting M1 Mϕ polarization in colitis. Furthermore, we found that $1,25(OH)_2D$ can regulate Mϕ phenotype switching by shifting the balance toward M2 and away from M1. Taken together, our data demonstrated that Mϕ phenotypes can be directly and significantly regulated by $1,25(OH)_2D$ in vitro, even though the effect was not as strong as predicted. This finding provides novel insight that vitamin D protects against colitis partially through regulating Mϕ phenotypes onsite. Further studies are needed to investigate the regulation of vitamin D on monocyte-Mϕ lineage at the stages prior to entering the inflamed colon, including monocyte production, migration and differentiation during experimental colitis progression.

4.2 Introduction

Inflammatory bowel disease (IBD) is a condition of chronic and relapsing inflammation in the gastrointestinal tract (*1*). Common symptoms of IBD include increased and sustained immune cell infiltration and pro-inflammatory cytokine production in the intestine (*2*). These chronic inflammatory responses cause intestinal tissue damage and delayed mucosa healing, which then increases IBD severity and duration (*3, 4*). Therefore, understanding the disease mechanisms and identifying conditions that control the gut immune response could be a promising IBD prevention and treatment strategy.

Macrophages (Mϕs) are crucial regulators of IBD development. Previous studies reported that dextran sodium sulfate (DSS)-induced colitis significantly increases the number of infiltrating Mϕs in the colon (*5*). However, Mϕs are a group of cells with high heterogeneity, and their role in colitis is tightly related to these phenotypes and their activation (*6-8*). Mature Mϕs can be polarized to two distinct phenotypes, M1 and M2, depending on the presence of environmental cytokines or inducers (*7-9*). The two phenotypes have distinct biological functions. M1 Mϕs produce pro-inflammatory mediators (e.g. TNF-α and ROS), that stimulate adaptive immune responses and induce tissue injury (*10*). In contrast, M2 Mϕs have anti-inflammatory features and facilitate tissue healing (*11*). These cell functions are also relevant to the DSS-colitis model. Following induction of colitis with DSS the ratio of M1 to M2 Mϕ increases to help eliminate infiltrating bacteria. After removal of DSS, the ratio decreases coincident with mucosal healing (*12, 13*). Therefore, manipulating Mϕ phenotypes during colitis may be a novel IBD management strategy.

Vitamin D is a crucial regulator of calcium absorption and bone health (*14*). However, recent evidence shows that it has anti-inflammatory roles in multiple types of immune cells including Mϕs, and in inflammatory diseases like IBD (*15*). Previous research found that vitamin D receptor (VDR) knockout mice develop more severe DSS-induced acute colitis and that M1 Mϕs related-cytokine production (e.g. TNF-α, IL-1α, IL-1β and IL-12) in the colon is increased. In addition, $1,25(OH)_2D$ administration reduces DSS-induced cytokine production in wild type mice (*16, 17*). In vitro studies also showed that 24 hours pretreatment of 20 nM $1,25(OH)_2D$ significantly suppresses LPS-induced pro-inflammatory cytokine production by mouse bone marrow derived Mϕs (BMDMs). Similarly, Korf et al. observed that 48 hours pretreatment of 10 nM $1,25(OH)_2D$ inhibited cytokine and chemokine gene expression of IFN-γ and LPS induced murine peritoneal Mϕs (*18, 19*). However, it is not known whether $1,25(OH)_2D$ can influence M1 and M2 Mϕ polarization or regulate M1 and M2 Mϕ phenotype switching when fully differentiated. In this study, we examined whether $1,25(OH)_2D$ can directly regulate Mϕ polarization and phenotype switch using cultured primary murine peritoneal Mϕs.

4.3 Materials and Methods

4.3.1 Reagents

Mϕ culture medium was made of 45% RPMI 1640 (Sigma R0883), 45% high glucose DMEM (GIBCO 11960), 10% FBS (Lonza), 100 U/ml Penicillin-streptomycin (Invitrogen 15140-122), 2 mM L-glutamine (Life Technology 25030) and 20 ng/ml murine M-CSF (R&D 416-ML-010). The culture medium was prepared within 1 week before use and stored at 4^oC.

1α,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D$) was purchased from Enzo Life Science. $1,25(OH)_2D$ stock was prepared in pure ethanol to a concentration of 10^{-3} M and stored in the dark at -80°C. 25x $1,25(OH)_2D$ working stocks (2.5, 25 and 250 mM) were freshly made by diluting 10^{-3} M $1,25(OH)_2D$ into Mϕ culture medium right before use. Direct light exposure to $1,25(OH)_2D$ was avoided during the preparation.

Lipopolysaccharides (LPS) was purchased from Sigma (Catalogue# L4391). LPS stock was prepared in RPMI 1640 supplemented with 10% FBS to a concentration of 1 mg/ml and stored at -80°C. 25x LPS working stock (25 ug/ml) was made by diluting 1 mg/ml LPS into Mϕ culture medium 1-7 days prior to use and stored at 4°C.

IFN-γ and IL-4 were purchased from Peprotech (Catalogue# AF-315-05 and AF-214-14). The 20 μg/ml stocks of both cytokines were prepared in RPMI 1640 supplemented with 10% FBS and stored at -80°C. 25x working stocks (500 ng/ml) were made by diluting 20 μg/ml cytokine stocks into Mϕ culture medium 1-7 days prior to use and stored at 4°C.

4.3.2 Animals

C57BL6/J mice were purchased from The Jackson Laboratories and a breeding colony was maintained in our lab. At weaning mice were group housed (4 mice/cage), given a standard chow diet and water ad libitum, and exposed to a 12-hour light/12-hour dark cycle in the Life Science Animal Facility at Purdue. All mouse experiments were approved by the Purdue Animal Care and Use Committee.

4.3.3 Peritoneal cavity cell isolation and culture.

Peritoneal Mφs were harvested from 6-8 week old mice (male and female) and cultured following published procedures with the following modification (*20*). Mice were

sacrificed with CO_2 and the peritoneal cavity cells were immediately collected by lavage using 5 ml cold RPMI 1640 per mouse. Each mouse yielded 2-4 million peritoneal cells, and the cells from 6-8 mice were pooled for each replicate of the experiments. Cells were kept ice cold throughout the harvest.

Isolated peritoneal cavity cells were centrifuged at 4^{o}C, 400 x g for 10 min and suspended into Mϕ culture medium (500 μl/mouse) (*20*). 10 μl cell suspension was mixed with an equal amount of 250 μg/ml trypan blue, and the viable cell number was determined using a hemocytometer. Peritoneal cavity cells were diluted to a concentration of 4 million/ml in the Mϕ culture medium. Cell suspensions were seeded into each well on a 24-well (500 μl/well) or 48-well (250 μl/well) cell culture plate. After 2-4 hours incubation (37^{o}C, 5% CO_2), floating cells were removed with three washes of RPMI 1640 (37^{o}C). Flow cytometric analysis of the adherent cells showed that about 90% of the cells are F4/80 and CD11b double positive peritoneal Mϕs (M0) (*20*). The purified Mϕs were allowed to rest for 1 hour before being treated to induce polarization.

4.3.4 Mϕ polarization and phenotype switching

In vitro Mϕ polarization was done following previously published procedures with modification (*20*). Instead of treatment the cells with IFN-γ and LPS together, M1 Mϕs polarization was induced by adding IFN-γ (final concentration: 20 ng/ml) for 1 hour followed by 5 hours co-treatment of IFN-γ with LPS (final concentration: 1 ug/ml). M2 Mϕ polarization was induced by 6 hour treatment of IL-4 (final concentration: 20 ng/ml). NOS2 and Arg1 gene expression levels were used to confirm M1 and M2 polarization, respectively.

To induce M1 Mϕ to switch to M2 Mϕ, polarized M1 Mϕ were washed 3 times with RPMI 1640, afterwhich they were treated with Mϕ culture medium containing 20 ng/ml IL-4. The gene expression ratio of Arg1 to NOS2 was measured to evaluate M1 to M2 phenotype switch. To induce M2 Mϕ to switch to M1 Mϕ, polarized M2 Mϕ were washed 3 times with RPMI 1640, afterwhich they were treated with Mϕ culture medium containing 20 ng/ml IFN-γ for 1 hour followed by 5 hour co-treatment of IFN-γ with LPS. The gene expression ratio of NOS2 to Arg1 was measured to evaluate M2 to M1 phenotype switch.

To assess the impact of $1,25(OH)_2D$ on Mϕ polarization and phenotype switching, the following experiments are designed.

4.3.5 Experiments design

Experiment 1. Test the effect of $1,25(OH)_2D$ alone on M0 polarization. M0 peritoneal Mϕs were cultured as described above. After washing off the floating cells, 500 μl Mϕ culture medium was added to each well on a 24-well cell culture plate. The various final concentrations of $1,25(OH)_2D$ (0, 0.1, 1 or 10 nM) were achieved by adding 10 μl culture medium or 25x $1,25(OH)_2D$ working stocks with different concentrations. After 6 hours incubation, cells were harvested for further analysis.

Experiment 2. Test the effect of $1,25(OH)_2D$ on IFN-r and LPS induced M1 polarization. Mϕs were maintained as M0 or polarized to M1 as previous described. In the M1 polarization groups, cells were co-treated with various concentrations of $1,25(OH)_2D$ (0, 0.1, 1 or 10 nM) from the beginning of the polarization for 6 hours.

Experiment 3. Test the effect of $1,25(OH)_2D$ on IL-4 induced M2 polarization. Mϕs were maintained as M0 or polarized to M2 as previous described. In the M2

polarization groups, cells were co-treated with various concentrations of 1,25$(OH)_2$D (0, 0.1, 1 or 10 nM) from the beginning of the polarization for 6 hours.

Experiment 4. Test the effect of 1,25$(OH)_2$D on M1 to M2 phenotype switch. Mϕs were maintained as M0 or polarized to M1 as previous described. At the end of 6 hours polarization, two thirds of M1 polarized cells were washed with 37^{o}C RPMI 1640 for three times. These cells were then incubated in Mϕ culture medium containing 20 ng/ml IL-4 with or without 10 nM 1,25$(OH)_2$D for 6 hours. Cells were then harvested for further analysis.

Experiment 5. Test the effect of 1,25$(OH)_2$D on M2 to M1 phenotype switch. Mϕs were maintained as M0 or polarized to M2 as previous described. At the end of 6 hours polarization, two thirds of M2 polarized cells were washed three times with 37^{o}C RPMI 1640. These cells were then incubated in Mϕ culture medium containing 20 ng/ml IFN-r with or without 10 nM 1,25$(OH)_2$D for 1 hour, and then co-treated with LPS for 5 hours to induce M1 class switch. Cells were then harvested for further analysis.

4.3.6 Mϕ harvest and gene expression analysis

Mϕs were washed three times with 37^{o}C RPMI 1640 at the end of the experiments and directly harvested into tri-reagent. Samples were collected in 1.5 ml screw cap tubes, snap frozen in liquid nitrogen and stored at -80^{o}C until use. RNA was isolated using Direct-zol RNA kits from Zymo research (catalogue No.R2050, Irvine, CA, USA). Purified RNA was reverse transcribed to make cDNA and analyzed for NOS2 and Arg1 gene expression; 18s ribosomal RNA (RNA18S) was used as a housekeeping gene. All primer sets were pre-made by Integrated DNA Technology (Assay ID: RNA18S:

Hs.PT.39a.22214856.g, NOS2: Mm.PT.58.43705194, Arg1: Mm.PT.58.8651372, Coralville, Iowa).

4.3.7 Statistical analysis

Each experiment included 3-5 analytical replicates of the pooled sample and all experiments were repeated at least three times. The data are expressed as the mean ± standard deviation (SD) and were analyzed with SAS Enterprise 5.1. In experiments 1-3, the gene expression levels of NOS2 and Arg-1 were normalized to the groups without $1,25(OH)_2D$ treatment, and the differences among groups were analyzed by one-way ANOVA followed by Fisher LSD multiple comparisons. In experiments 4 and 5, the difference of gene expression or ratio between groups were analyzed by the student's t-test. A difference was considered significant if the p value was less than 0.05.

4.4 Results

Arg1 marks the polarization of M2 Mϕ phenotype. Arg1 mRNA levels were increased by 35% after 6 hours treatment with 1 nM $1,25(OH)_2D$ (Figure 4.1), but higher doses (10 nM) had no additional effect. This suggests that $1,25(OH)_2D$ alone can induce M2 Mϕ polarization.

M0 Mϕs had undetectable NOS2 expression. Treatment with IFN-γ and LPS significantly elevated NOS2 mRNA levels (Figure 4.2), indicating that the M0 Mϕs were successfully polarized to a M1-like phenotype. While 0.1 nM $1,25(OH)_2D$ did not block IFN-γ/LPS-induced NOS2 mRNA induction, high doses of $1,25(OH)_2D$ treatment (1 and 10 nM) significantly suppressed induction of NOS2 mRNA by 20% (Figure 4.2).

Treatment of M0 Mϕs with IL-4 increased Arg1 mRNA level by 5.5 fold (Figure 4.3), indicating a successful induction of the M2-like polarization. Co-treatment of IL-4

with 1,25$(OH)_2$D significantly increased Arg1 gene expression by 30% only at the 10 nM 1,25$(OH)_2$D dose (Figure 4.3).

Incubating M1-like Mϕs with 20 ng/ml IL-4 for 6 hours significantly reduced NOS2 mRNA level and increased Arg1 mRNA level (Figure 4.4 A-B) leading to an increased Arg1/NOS2 gene expression ratio, indicating a successful induction of the M1-to-M2-phenotype switch (Figure 4.4 C). Co-treatment of 10 nM 1,25$(OH)_2$D with IL-4 further reduced NOS2 expression levels by 20% but did not alter Arg1 mRNA levels (Figure 4.4 A-B). This suggests that 1,25$(OH)_2$D may only have a mild effect on inducing M1-to-M2 phenotype switch (Figure 4.4 C).

IFN-γ and LPS treatment significantly increased NOS2 and reduced Arg1 mRNA levels in M2-like Mϕs (Figure 4.5 A-B), resulting in a significant increase of the NOS2/Arg1 mRNA ratio (Figure 4.5 C). This indicated a successful induction of M2 to M1-like Mϕ phenotype switch. Co-treatment with 10 nM 1,25$(OH)_2$D significantly reduced NOS2 mRNA levels, but did not significantly increase Arg1 mRNA levels (Figure 4.5 A-B). 1,25$(OH)_2$D reduced the NOS2/Arg1 mRNA ratio by 40% (Figure 4.5 C), indicating a moderate inhibitory effect of 1,25$(OH)_2$D on the M2-to-M1 phenotype switch.

4.5 Discussion

Here we conducted a series of experiments using primary mouse peritoneal Mϕ models and confirmed that Mϕ phenotypes are directly regulated by 1,25$(OH)_2$D. Specifically, 1,25$(OH)_2$D suppressed expression of the M1-like Mϕ marker NOS2 but induced expression of the M2-like cell marker Arg-1. This suggests that 1,25$(OH)_2$D can inhibit M1 polarization and facilitate M2 polarization. Consistent with this conclusion,

$1,25(OH)_2D$ can also inhibit the M2-to-M1 phenotype switch and induce M1 to polarize to M2 Mϕ with mild to moderate effects.

Studying the mechanism of $1,25(OH)_2D$ regulation on Mϕ polarization and phenotype switching has a significant implications for the management of human IBD. As what has been discussed in previous chapters, low vitamin D status is often diagnosed in human IBD patients, and mice with low vitamin D intake or VDR deletion are more susceptible to developing experimental colitis (*16, 21-23*). Vitamin D has been proposed to have anti-colitis effects while its regulating targets -Mϕs- are proved to play a central role in experimental colitis and human IBD progression (*12, 13*). Froicu et al. showed that mice with global VDR deletion developed more robust colonic inflammation and had increased M1 Mϕ cytokine levels (e.g. TNF-α, IL-1β and IL-12) when treated with DSS, but the mice were not fed rescue diet so the effect caused by VDR KO may also be due to altered calcium metabolism (*16*). By recovering VDR in the intestine using HV2; VDR KO mice (chapter 3), our study excluded the co-factor of calcium and independently confirmed that the M1 Mϕ cytokine levels were significantly elevated in HV2; VDR KO mice, and that this increase was associated with delayed mucosa healing after DSS induced colon damage. The elevated M1 response in inflamed colon may be due to increased total Mϕ number in the colon, or due to altered Mϕ phenotype. It has been long considered that the Mϕ infiltrating number in the gastrointestinal tract indicates the disease stage, because increased pro-inflammatory Mϕs numbers were observed in the colon of both DSS-colitis and human IBD (*5, 24*). However, this viewpoint has been challenged as the Mϕ phenotypes and functions are linked to IBD progression. During gut inflammation, circulating monocytes are recruited to the intestine and further

differentiate into either classically activated Mϕ (M1) or alternatively activated Mϕ (M2), depending on the environmental cytokine profiles (*10, 25*). M1 Mϕs are polarized by LPS and IFN-γ and characterized as pro-inflammatory Mϕs. They produce large amounts of pro-inflammatory mediators (TNF-α, IL-1, IL-12 and ROS) to stimulate local immune response and induce tissue damage (*10*). M2 Mϕs are anti-inflammatory Mϕs induced by IL-4 or IL-13 (*25*). Unlike M1, M2 cells produce high levels of IL-10 and growth factors (e.g. TGF-β) to inhibit M1 activity and facilitate mucosa healing (*11*). Collectively, M1 Mϕs are believed to be detrimental in colitis especially during mucosa healing, while M2 Mϕs have a protective effect during the disease progression (*12, 13*). Therefore, it is essential to demonstrate the contribution of vitamin D on Mϕ phenotype regulation and I hypothesize that the increased M1 response seen in HV2; VDR KO mice colon is due to unbalanced M1 and M2 Mϕ phenotype.

Early evidence showed that 50 nM $1,25(OH)_2D$ can induce bone marrow derived Mϕ differentiation with increased cell surface expression of M2 marker mannose receptor after 2-4 days culture (*26*). Later on, $1,25(OH)_2D$ (10 nM, 3 days) was used to differentiate M2-like cells from the human monocyte cell line THP-1 (*27*). Unfortunately, these studies were conducted either before the concept of M2 Mϕ coming into place or without sufficient details about cell polarization due to different research goals (vitamin D and monocyte to Mϕ differentiation). Therefore, the question of whether vitamin D can directly regulate M2 Mϕ polarization still requires further investigation. Our study independently showed that $1,25(OH)_2D$ alone significantly induced M2 marker Arg1 gene expression level in peritoneal steady stage Mϕs, but the mechanism for this effect is unknown. In vitro M2 Mϕ polarization requires IL-4 to activate the JAK-STAT6

pathway, which then induces Arg1 expression (*28*). In the EAE mouse model of multiple sclerosis, vitamin D increases Th cell STAT6 gene expression and enhances Th2 polarization (*29*). Since STAT6 is also expressed in Mϕs and drives IL-4 induced M2 polarization, we hypothesize that $1,25(OH)_2D$ can induce M2 polarization by regulating the same pathway(*30*). Our results showed that $1,25(OH)_2D$ increased IL-4 induced Arg1 expression in M2-like Mϕ, which was consistent with our hypothesis. However, further experiments are required to determine whether STAT6 is a direct $1,25(OH)_2D$ target gene or whether JAK-STAT6 signaling is affected in Mϕs by $1,25(OH)_2D$.

$1,25(OH)_2D$ is an immune modulator which can suppress Mϕ pro-inflammatory response. $1,25(OH)_2D$ suppresses a variety of pro-inflammatory cytokines in human monocyte/Mϕs cell lines (*31-33*), and a similar immune-suppressive effect was also seen in mouse Mϕ cell lines (e.g. P388D1) and primary cell cultures (e.g. BMDM and peritoneal Mϕ) (*18, 19, 34*). For example, Korf et al. showed that 48 hours pretreatment of 10 nM $1,25(OH)_2D$ significantly inhibited IFN-γ and LPS induced M1 gene expression levels (e.g. NOS2 and TNF-α) of mouse peritoneal Mϕs (*18, 19*). In addition, 24 hours co-treatment of $1,25(OH)_2D$ suppressed IFN-γ induced oxidative burst and host defense mediators in murine BMDMs (*35*). Mechanistically, $1,25(OH)_2D$ was found to suppress NF-κb and MAPK/AP-1 pathways to inhibit Mϕ pro-inflammatory response (*18, 19, 28, 34, 36, 37*). For example, Cohen-Lahav et al. demonstrated that pre-incubation with 100 nM $1,25(OH)_2D$ significantly reduced LPS stimulated TNF-α production from mouse Mϕ cell line P388D1 by prolonging Iκbα-mRNA half-life and by decreasing Iκbα phosphorylation (*34*). This may also explain the inhibition of p65 nucleus translocation by $1,25(OH)_2D$ (*34*). 10 nM $1,25(OH)_2D$ significantly inhibited LPS-induced IL-6 and

TNF-α production by human monocytes and mouse BMDM with increased MAPK phosphatase-1 level, while the 1,25$(OH)_2$D effect was significantly reduced when using BMDM from MAPK phosphatase-1 knockout mice to repeat the experiment (*36, 37*). To fit within the concept of Mϕ polarization, our study showed that 6 hours 1,25$(OH)_2$D treatment was sufficient to inhibit IFN-γ and LPS induced M1 Mϕ polarization by suppressing Mϕs NOS2 expression levels.

Other studies have demonstrated that M1 and M2 phenotypes can be stimulated to switch phenotypes (*9, 38*), and this has been independently confirmed in our study. In DSS-induced experimental colitis mouse model, the ratio of M1 to M2 Mϕ number increased with colitis severity, while the ratio decreased during disease recovery and mucosa healing (*12, 13*). Similarly, in humans, IBD patients treated with infliximab or together with azathioprine (anti-TNF-α antibodies) had reduced total Mϕ numbers ($CD68^+$) and increased M2 Mϕ ($CD206^+$) numbers in mucosa that was followed by improved mucosa healing (*39, 40*). A recent study using RAW 264.7 cell line showed that 1,25$(OH)_2$D significantly induced high glucose stimulated M1-like Mϕ s to class-switch to a M2 phenotype; this effect is mediated through upregulating the PPARγ signaling pathway (*41*). Our study used IFN-γ and LPS as M1 stimuli, and independently showed that 1,25$(OH)_2$D significantly suppressed IL-4 polarized M2 Mϕs to switch to a M1 phenotype. By changing the M1 stimuli in the culture system, our study may better mimic the impact of 1,25$(OH)_2$D on Mϕ phenotype switching in the microenvironment of inflamed colon induced by DSS.

Even though the regulation of Mϕ phenotypes by 1,25$(OH)_2$D has been demonstrated here in vitro, whether this is also the scenario in colitis mouse models with

vitamin D signaling deficiency remains unknown. Future studies need to address this question to emphasize its clinical implication. Moreover, the modulating effect of $1,25(OH)_2D$ is much milder than that of the M1 or M2 Mϕ polarization inducers. Compared to the dramatic effect of immune cell VDR deletion caused severe colitis described in the previous chapter, the $1,25(OH)_2D$-induced mild to moderate local Mϕ polarization regulation is not sufficient to explain the observation in vivo. Further studies also need to determine the effect of vitamin D signaling in monocyte generation, migration and monocyte to Mϕ differentiation during colitis.

4.6 Conclusions

The current study demonstrated that $1,25(OH)_2D$ can suppress M1 Mϕ polarization while facilitating M2 Mϕ polarization. Meanwhile, we also found that $1,25(OH)_2D$ has a significant impact on inhibiting M2 to M1 class switch in vitro. Taken together, our findings contribute to the understanding of the colonic protective effect of vitamin D in IBD and provide novel insights into IBD management. These results may also shed light on other diseases with inflammation caused tissue injury.

4.7 Acknowledgement

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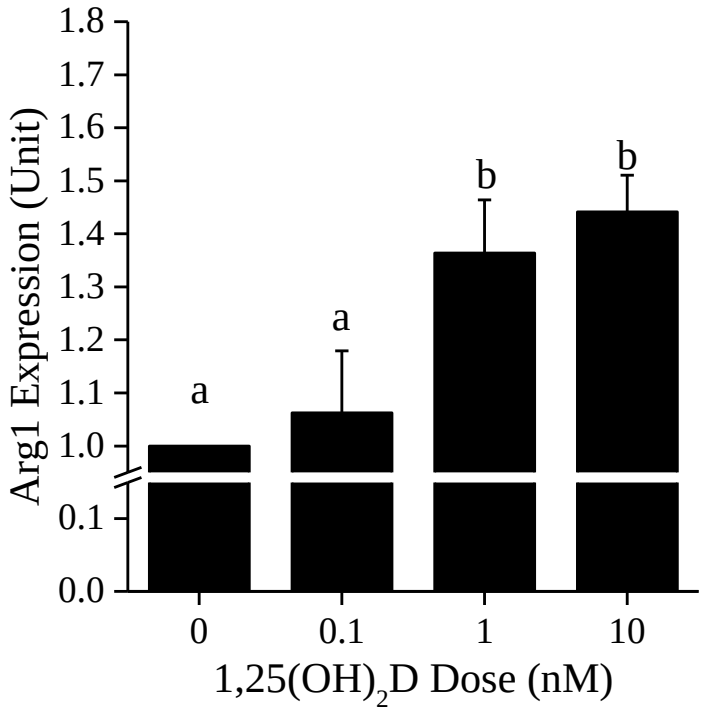

Figure 4.1 Arg1 gene expression levels in steady M0 peritoneal macrophages treated with different doses of $1,25(OH)_2D$. Data was normalized to 0 nM $1,25(OH)_2D$ treatment group. (1,25D, $1,25(OH)_2D$; Different letters, $p<0.05$).

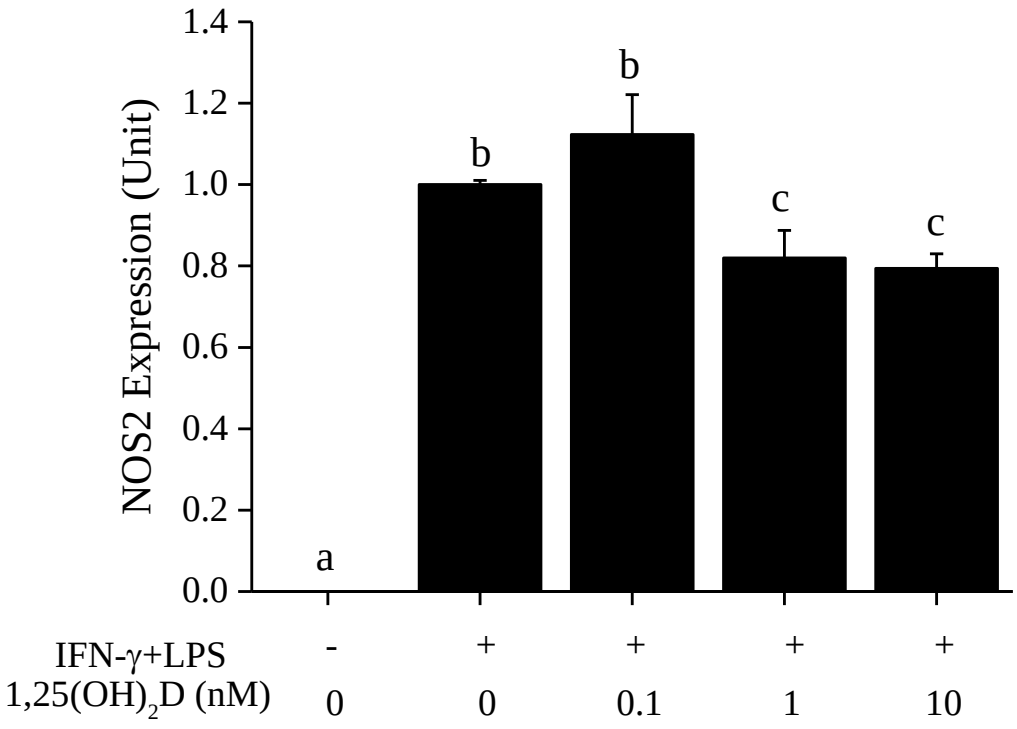

Figure 4.2 NOS2 expression levels in peritoneal M0 or M1 Mϕs treated with different doses of $1,25(OH)_2D$. Data was normalized to 0 nM $1,25(OH)_2D$ treated M1 polarization group. (1,25D, $1,25(OH)_2D$; Different letters, $p<0.05$).

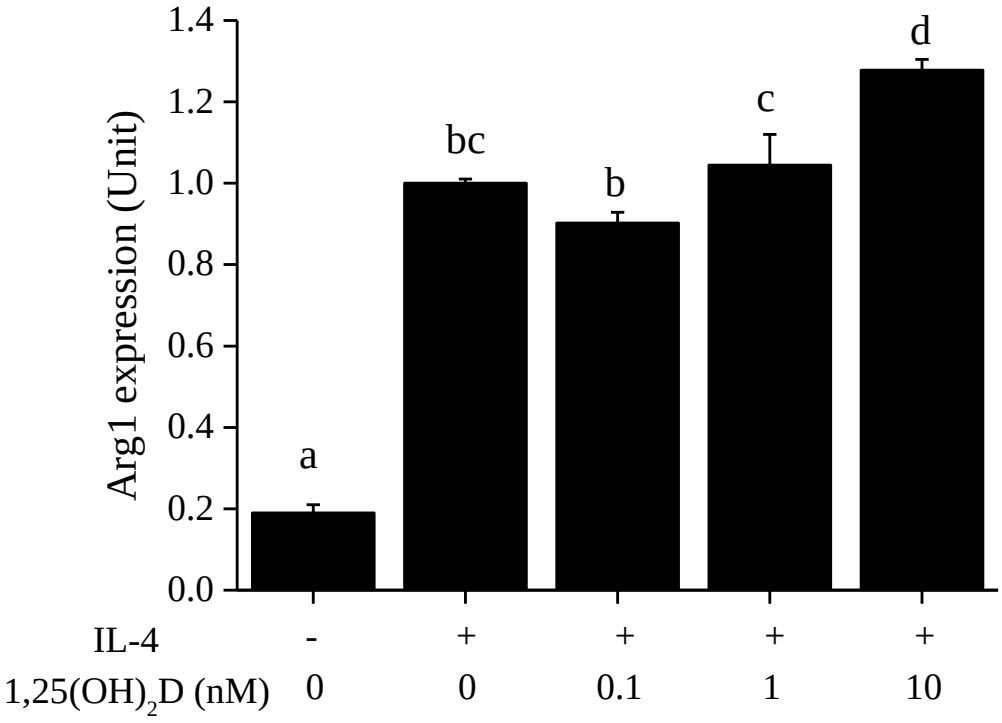

Figure 4.3 Arg1 expression levels in peritoneal M0 or M2 Mϕs treated with different doses of $1,25(OH)_2D$. Data was normalized to 0 nM $1,25(OH)_2D$ treated M2 polarization group. (1,25D,$1,25(OH)_2D$; Different letters, $p<0.05$).

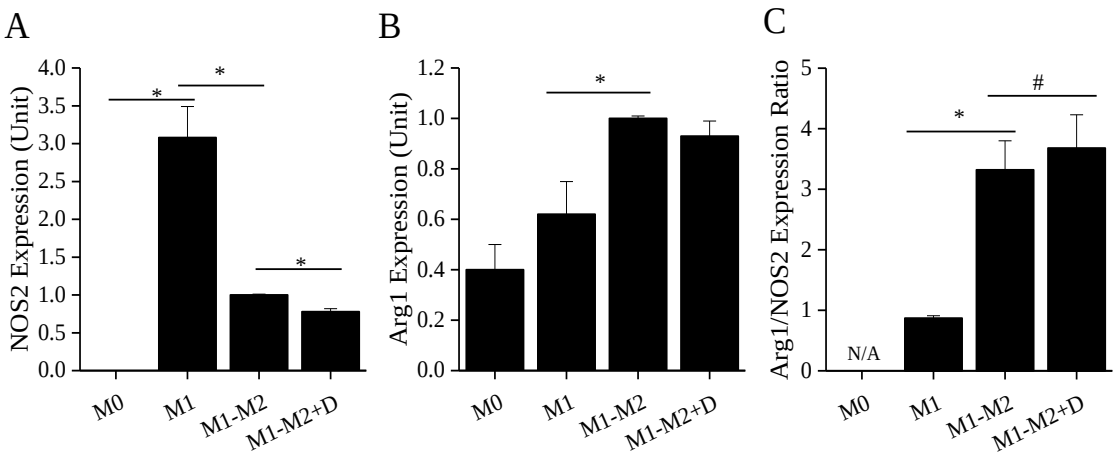

Figure 4.4 Gene expression levels of NOS2 and Arg1 during Mϕ M1 to M2 phenotype switching in the presence or absence of $1,25(OH)_2D$. NOS2 gene expression (A), Arg1 gene expression (B) and Arg1/NOS2 expression ratios of peritoneal Mϕs with different treatment. M0, non-polarized peritoneal Mϕs. M1, IFN-γ and LPS polarized peritoneal Mϕs. M1-M2, M1 Mϕs were induced for M2 phenotype switch by culturing them with IL-4 for 6h. M1-M2+D, both IL-4 and 10 nM $1,25(OH)_2D$ were used for M2 phenotype switch. NOS2 and Arg1 expression levels were normalized to M1-M2 group. Arg1/NOS2 gene expression ratios were calculated from raw data. (D, $1,25(OH)_2D$ *, $p<0.05$; #, $p<0.10$).

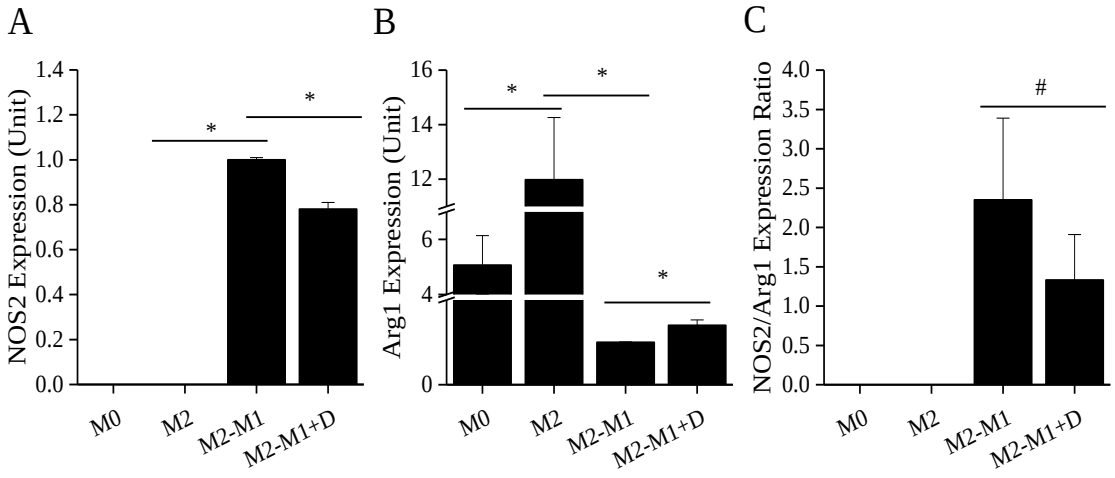

Figure 4.5 Gene expression levels of NOS2 and Arg1 during Mϕ M1 to M2 phenotype switching in the presence or absence of 1,25(OH)$_2$D. NOS2 gene expression (A), Arg1 gene expression (B) and NOS2/Arg1 expression ratios of peritoneal Mϕs with different treatment. M0, non-polarized peritoneal Mϕs. M2, IL-4 polarized peritoneal Mϕs. M2-M1, M2 Mϕs were induced for M1 phenotype switch by culturing them with IFN-γ and LPS. M2-M1+D, 10 nM 1,25(OH)$_2$D was used as co-treatment when inducing M1 phenotype switch. NOS2 and Arg1 expression levels were normalized to M2-M1 group. NOS2/Arg1gene expression ratios were calculated from raw data. (D, 1,25(OH)$_2$D; *, $p<0.05$; #, $p<0.10$).

CHAPTER 5. RESEARCH SUMMARY AND FUTURE DIRECTIONS

5.1 Research Summary

My thesis work aimed to achieve two major research goals. The first was to develop a mouse model for studying the colon epithelial cell gene function in colitis, and the second was to investigate the cell targets and mechanisms of vitamin D-mediated protection against colitis. Here I will summarize the main findings regarding these two goals, as detailed in the previous chapters.

The study reported in chapter 2 described a novel mouse model for colitis and colitis-associated colon cancer research. The utility of this model was originally reported in 2010 and then was expanded by several means (*1, 2*). First, the Cre-mediated transgene induced gene recombination was not only restricted in the large intestine, but also expanded after DSS treatment. This allowed us to test the gene function in colon epithelial cells during colitis without confounding factors from other tissues. Second, this expansion was strongly correlated with epithelial damage and was sustained during and after the epithelial healing. This made it possible to measure the gene function specifically in mucosa healing. Even though the mechanism of the reported phenomena is unknown, we hypothesized that the colonic stem cells are affected because of the involvement of crypt base cells and their long lasting effect. Stem cell lineage tracing is

required to further identify the contribution of a specific group of stem cells to mucosa healing and their transgene activity during DSS induced colitis in mice.

With the promising model developed, we then investigated the effect of colon epithelial cell VDR on DSS-induced colitis prevention. We found that even though colon epithelial cell VDR deletion resulted in more severe colon damage induced by DSS, it was not sufficient to cause delayed mucosa healing, increased colonic cytokine expression or more robust systemic colitis response. On the other hand, non-epithelial cell VDR KO mice recaptured all the local and systemic colitis symptoms seen in vitamin D deficiency and VDR KO, including increased colonic damage and inflammation, delayed healing, more robust body weight loss, spleen enlargement and DAI. Therefore, we conclude that colon epithelial cell VDR is not sufficient to prevent colitis development or maintain mucosa healing.

Next I investigated the mechanism of non-epithelial cell VDR deletion that caused delayed mucosa healing and severe colitis. I specifically asked the question of whether monocyte-Mϕ lineage is the primary vitamin D regulating target. Three lines of evidence support this hypothesis. First, in the colon tissue of mice with non-epithelial cell VDR deleted, pro-inflammatory Mϕ cytokine expression levels were significantly elevated during the mucosa healing stage after DSS treatment. However, this was not seen in the colon epithelial cell VDR KO mice. Second, Mϕ marker NOS2 was significantly induced in the spleen of non-epithelial cell VDR KO mice but not in colon epithelial cell VDR KO mice. Third, spleen red pulp, where monocytes and most Mϕs are located, was significantly enlarged in non-epithelial cell VDR KO mice. These data suggest that

vitamin D mediated monocyte-Mϕ lineage response strongly affects DSS-induced experimental colitis in mice.

Figure 5.1 summarizes this cell lineage response during experimental colitis and potential vitamin D regulating steps, including monocyte differentiation in bone marrow, emigration to blood circulation, migration to colon, Mϕ polarization in inflamed colon and their interaction with T cells. I first hypothesized that vitamin D can directly modulate Mϕ polarization and phenotype switch away from M1 and towards M2 in the colon during colitis. This hypothesis was partially supported by the experiments I performed in chapter 4 using primary Mϕ culture. I found that $1,25(OH)_2D$ treatment significantly suppressed M0 to M1 polarization and M2 to M1 phenotype switching in the pro-inflammatory environment, and this also fit with the observation in HV2; VDR KO mice that deleting VDR from immune cells elevated M1 response. $1,25(OH)_2D$ also induced M2 polarization in the presence or absence of IL-4. Even though $1,25(OH)_2D$ modulates Mϕs phenotypes, it is much less effective than the cytokine inducers. Moreover, the effect seen in cell culture experiments (Chapter 4) was much less dramatic than that in the colon of HV2; VDR KO mice (chapter 3). Therefore, I hypothesize that other mechanisms may still exist to contribute to the colitis response seen in HV2; VDR KO mice, including the upstream regulation on monocyte migration.

5.2 Future Directions

Previous experiments reported in this thesis mainly focused on potential vitamin D mediated Mϕ polarization and activation in the colon tissue, but the increased M1 Mϕ response in the inflamed colon seen in HVV2; VDR KO mice may also be due to

upstream regulation in blood circulation and in bone. To further investigate the mechanism of vitamin D mediated protection from colitis through monocytes and Mϕs requires the following questions to be answered.

Question 1: Does Vitamin D signaling prevent monocyte migration from blood circulation into the inflamed colon?

Rationale: Mϕs in the inflamed colon are differentiated from blood monocytes after their migration to the tissue (*3*). The monocyte migration process is strongly dependent on the MCP-1/CCR2 pathway (*3*). MCP-1 is produced by multiple cell types in the colon when it is infected, including blood vessel endothelial cells, colon epithelial cells, stromal cells and others (*4*). Among these cell types, Mϕs are the most significant producers of MCP-1(*4*). CCR2 is expressed on the cell surface of monocytes, which receives the MCP-1 signaling and induces the migration event. Vitamin D signaling may inhibit monocyte migration to local tissue by reducing colonic MCP-1. Evidence showed that colonic MCP-1 expression was increased in mice with DSS induced colitis, and further elevated in mice with intestinal VDR deletion (*5*, *6*). In AOM/DSS induced colon cancer mouse model, VDR deletion increased both the tumor burden in the colon and the MCP-1 expression level in the tumor (*7*). In vitro, LPS induced MCP-1 production by THP-1 cells and THP-1 induced Mϕs, and this pro-inflammatory effect was significantly suppressed by $1,25(OH)_2D$ pre-treatment in both groups of cells (*8*). This suggests that $1,25(OH)_2D$ may directly regulate monocyte-Mϕ MCP-1 expression and thus induce monocyte migration to the inflamed colon.

Hypothesis: vitamin D signaling inhibits monocyte migration from blood circulation to inflamed colon by suppressing MCP-1 production in DSS-colitis mice.

Experiments: In our experiments discussed in chapter 3, the increased Mϕ pro-inflammatory response in HV2; VDR KO mouse colon may be due to elevated colonic MCP-1 level and total Mϕ number. To test the hypothesis, MCP-1 gene expression levels in both CAC; VDR KO and HV2; VDR KO mice should be measured and I expect to see increased MCP-1 only in HV2; VDR KO mice colon. Total Mϕ numbers in the colon of HV2; VDR KO and control mice should be measured by immunohistochemical staining of CD68. I expect to see increased Mϕ numbers in the colon of HV2; VDR KO mice. Furthermore, the direct regulation of $1,25(OH)_2D$ on monocyte migration in vitro should be tested using transwell culture system.

Question 2: Can vitamin D signaling suppress monocyte emigration ability from bone marrow during colitis?

Rationale: Systemic inflammation induces bone marrow monocyte production and emigration and thus increases circulating monocyte numbers. Monocyte emigration from bone marrow is also dependent on the MCP-1/CCR2 pathway (*9*). As mentioned before, monocytes express high levels of CCR2 on the cell surface and monocytes CCR2 deletion caused their accumulation in the bone marrow after LPS injection in mice (*9*). Vitamin D was proposed to have anti-migration effects partially through its suppression of CCR2 expression on monocytes/ Mϕs (*6, 10*). MCP-1 is the chemokine ligand of CCR2 which is mainly produced by monocytes in the blood (*4*). At steady stage, serum MCP-1 ensures that newly differentiated monocytes emigrate into the circulation and

replenish the blood monocyte population. This MCP-1 level was elevated in mice fed western diet containing low vitamin D and calcium (*11*). Serum MCP-1 concentration is positively correlated with circulating $CD11b^+$ monocyte numbers in mice (*12*) and is upregulated in both DSS and TNBS induced experimental colitis model in mice (*5, 13*). However, whether low vitamin D signaling induces serum MCP-1 levels or monocyte emigration from bone marrow to blood circulation remains unknown.

Hypothesis: vitamin D signaling inhibits MCP-1 induced monocytes emigration from bone marrow to blood circulation during DSS-colitis.

Experiments: First I will test the serum MCP-1 concentration in HV2; VDR KO and control mice with DSS-colitis in chapter3, and I expect to see that the MCP-1 serum levels are high in HV2; VDR KO mice. I also want to test the total blood monocyte ($Ly6C^+CD11b^+$) numbers in HV2; VDR KO mice and expect to see elevated numbers compared to controls. Since blood monocytes are the major source of serum MCP-1, I will also measure the MCP-1 production in vitro from LPS stimulated murine monocytes with or without $1,25(OH)_2D$, and I expect to see reduced monocyte MCP-1 production with $1,25(OH)_2D$ treatment.

Question 3: Does VDR in monocytes and Mϕs protect mice from experimental colitis?

Rationale: Several lines of evidence showed that monocytes and Mϕs are the essential immune regulators during colitis. As described in chapter 1, systemic infection increased bone marrow monocyte production and Mϕ phenotype balance was tightly related to DSS-colitis progression and recovery (*14, 15*). In addition, T cell mediated colitis in mice can be alleviated by transferring M2 Mϕs into the peritoneum (*16*). Since

vitamin D can potentially regulate monocyte-Mϕ biology at different stages, I hypothesize that VDR in monocyte –Mϕ lineage has a protective role in colitis development. To test this hypothesis, one needs to induce colitis in a mouse model with monocyte-Mϕ VDR deletion. Currently, three mouse models have been developed to study myeloid cell biology with Cre recombinase expressing in monocytes and Mϕs (*17*). The first model is LysMCre. LysMCre mouse model has Cre transgene expression in the myeloid cell lineage, including monocytes and mature Mϕs, but also granulocytes (e.g. neutrophils) (*18*). Therefore the gene function observed from this model will have a confounding effect from neutrophils. The second model is Cx3cr1-cre or Cx3cr1-creER (*19*). These mice have transgene expression in most of the tissue resident Mϕs, but these resident Mϕs are not our main focus in DSS-colitis. The third model is FVB-Tg(Csf1r-icre)1Jwp/J (cfms-icre) (*20*). CSF-1 signaling is crucial for monocyte lineage development in bone marrow and Csf1r is expressed in different stages of monocyte precursors. Using cfms-icre to inactivate VDR will cause confounding effects from dendritic cells and bone marrow derived granulocytes derived from those precursors. To overcome the non-specificity, an inducible model Tg(Csf1r-Mer-iCre-Mer)1Jwp was developed to limit the recombination event in blood monocytes (*21*) . The disadvantage of this model is that the Cre transgene activation requires daily injection of 4-hydroxytamoxifen which increases the complexity and variation of the experiments (*22*). Given these considerations, we will use the LysMCre model for future experiments.

Hypothesis: VDR signaling in monocyte-Mϕ lineage protects against DSS induced colitis in mice.

Experiments: Mice with monocyte-Mϕ lineage VDR deletion (LysMCre-VDR KO) can be generated by crossing LysMCre with *VDR*$^{\Delta ex2/\Delta ex2}$ breeders. DSS-colitis will be induced as described in chapter 3. I hypothesize that LysMCre-VDR KO mice will develop more severe systemic and colonic response in DSS-colitis. I expect to see that LysMCre-VDR KO mice have 1) more severe body weight loss, DAI and spleen enlargement; 2) increased serum MCP-1 concentration and $Ly6C^+CD11b^+$ cell number in the circulation; and 3) increased total Mϕ number and elevated ratio of M1 to M2 Mϕ cell number/response in the colon.

Question 4: Is MCP-1 a direct VDR target gene?

Rationale: Previous research showed that 0.2 μg/ml LPS induced MCP-1 production in THP-1 cells can be significantly suppressed by 24-48 hours treatment of 100 nM $1,25(OH)_2D$ (*8*). More interestingly, 4 hours $1,25(OH)_2D$ treatment was sufficient to suppress THP-1 cell MCP-1 gene expression by over 50% without LPS stimulation (*23*). This quick response suggested a direct regulation of $1,25(OH)_2D$ on MCP-1 gene. To investigate whether MCP-1 is a vitamin D target gene in THP-1 cells, the authors also did FAIRE-seq, VDR ChIP-seq and CTCF ChIP-seq and found the overlapping regions among the analysis (*23*). This suggests that VDR can directly regulate MCP-1 gene transcription but further experiments are needed to identify the functional VDREs. Since mouse models are widely used to study human colitis, it is also crucial to determine whether MCP-1 is a direct regulating target in primary mouse monocyte-Mϕ lineage. It is also important to identify potential murine and human functional VDREs in MCP-1 gene promoter and enhancer regions to make further comparisons across the two species.

Hypothesis: MCP-1 is a vitamin D target gene.

Experiments: First I need to confirm that 1,25$(OH)_2$D treatment can also suppress MCP-1 gene expression in murine monocytes and Mϕs with or without LPS stimulation. Next I will do bioinformatics analysis to identify putative VDRE sequences in the MCP-1 promoter and enhancer regions. The ENCODE project portal (http://genome.ucsc.edu/ENCODE/) will be used to identify DNase I hypersensitive sites (HSS) in the MCP-1 mouse gene. Putative VDREs within these HHS will be identified with Lasagna-Search 2.0. EMSA and Chromatin immunoprecipitation (ChIP) assay will then be performed to test the binding capacity of VDR to the identified putative VDREs. If the binding capacity of VDR for these putative VDREs is demonstrated in the promoter region, a reporter gene assay will be used to test the function of these VDREs. If the putative VDREs are identified in the enhancer regions, a Chromatin Conformation Capture experiment will be used to test the function of the VDREs.

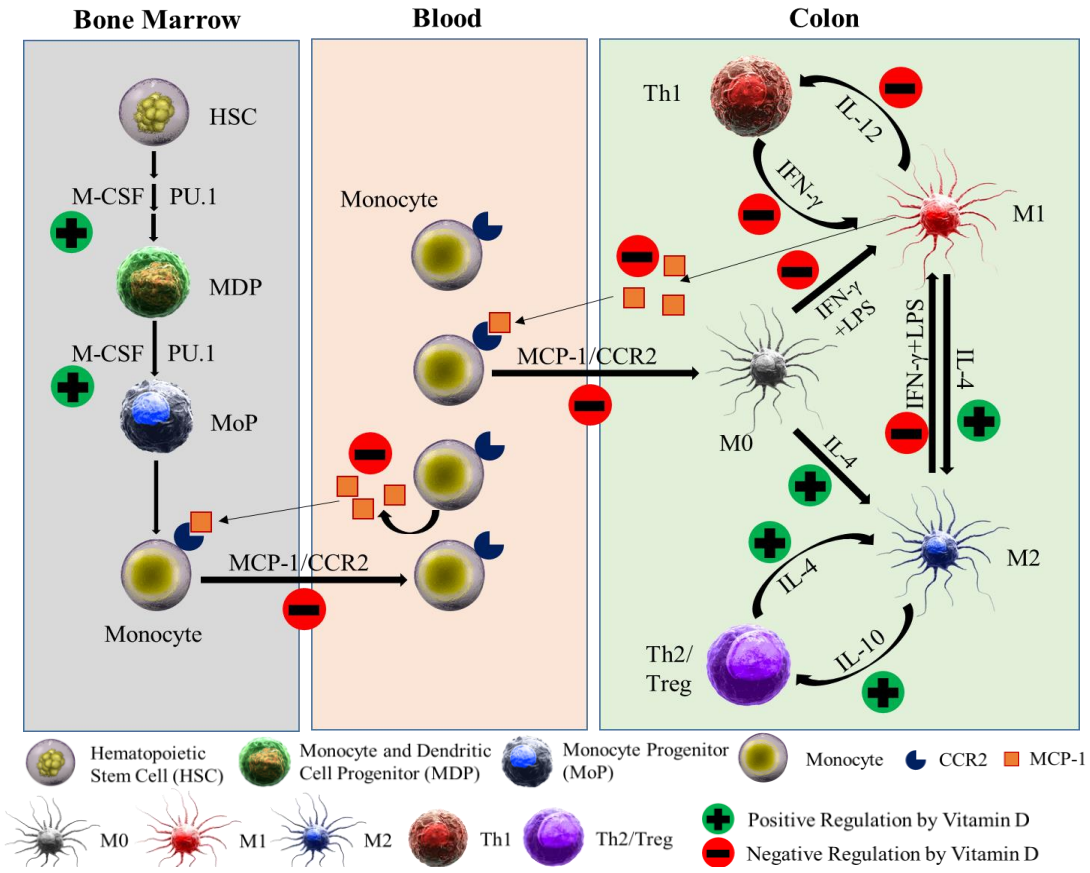

Figure 5.1 Monocyte-Mϕ lineage response during experimental colitis and potential vitamin D regulating targets. Bone marrow: monocytes are differentiated from bone marrow. This process is M-CSF dependent and potentially upregulated by vitamin D. Monocyte emigration from bone marrow to blood circulation is MCP-1/CCR2 dependent and this is potentially inhibited by vitamin D. Blood: monocytes circulating in blood produce increased level of MCP-1 during DSS-colitis, which induced the cell emigration from bone marrow to blood. Blood monocytes also migrate to inflamed colon and this process is also regulated by MCP-1/CCR2 signaling. Colon: migrated monocytes will differentiate into M1 or M2 Mϕs depending on the cytokine profile in the colon microenvironment. The differentiated M1 or M2 Mϕ phenotypes are also interchangeable. Colonic Mϕs can also interact with T cells during colitis.

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VITA

VITA

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